Selective Targeting of Cyclin E1-Amplified High-Grade Serous Ovarian Cancer by Cyclin-Dependent Kinase 2 and AKT Inhibition

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Abstract

Purpose: Cyclin E1 (CCNE1) amplification is associated with primary treatment resistance and poor outcome in high-grade serous ovarian cancer (HGSC). Here, we explore approaches to target CCNE1-amplified cancers and potential strategies to overcome resistance to targeted agents.

Experimental Design: To examine dependency on CDK2 in CCNE1-amplified HGSC, we utilized siRNA and conditional shRNA gene suppression, and chemical inhibition using dinaciclib, a small-molecule CDK2 inhibitor. High-throughput compound screening was used to identify selective synergistic drug combinations, as well as combinations that may overcome drug resistance. An observed relationship between CCNE1 and the AKT pathway was further explored in genomic data from primary tumors, and functional studies in fallopian tube secretory cells.

Results: We validate CDK2 as a therapeutic target by demonstrating selective sensitivity to gene suppression. However, we found that dinaciclib did not trigger amplicon-dependent sensitivity in a panel of HGSC cell lines. A high-throughput compound screen identified synergistic combinations in CCNE1-amplified HGSC, including dinaciclib and AKT inhibitors. Analysis of genomic data from TCGA demonstrated coamplification of CCNE1 and AKT2. Overexpression of Cyclin E1 and AKT isoforms, in addition to mutant TP53, imparted malignant characteristics in untransformed fallopian tube secretory cells, the dominant site of origin of HGSC.

Conclusions: These findings suggest a specific dependency of CCNE1-amplified tumors for AKT activity, and point to a novel combination of dinaciclib and AKT inhibitors that may selectively target patients with CCNE1-amplified HGSC. Clin Cancer Res; 23(7): 1862–74. ©2016 AACR.

Introduction

Targeted therapies have changed the management of many cancers types, resulting in significant improvements in clinical response rates and survival (1). However, while the antiangiogenic mAb bevacizumab (2, 3) and the PARP inhibitor olaparib (4, 5) have entered care in high-grade serous ovarian cancer (HGSC) recently, the development of targeted therapy to this disease has been relatively slow.

HGSCs are characterized by ubiquitous TP53 mutations, genomic instability, and widespread copy number alterations, with relatively infrequent somatic point mutations of driver genes (6, 7). Structural aberration also contributes to loss of tumor suppressors such as RB1 and NF1 by gene breakage (8). Defects in the homologous recombination repair (HR) pathway are present in approximately 50% of HGSCs, primarily associated with germ-line and somatic mutations in BRCA1, BRCA2, and associated proteins (7). HR deficiency imparts platinum sensitivity in HGSC, and provides the basis for the use of PARP inhibitors that target compensatory DNA repair pathways (4, 9). Of HGSC with intact HR, amplification of CCNE1, which encodes the cell-cycle regulator cyclin E1, is the best characterized driver. CCNE1 amplification or gain occurs in 20% of all HGSC tumors and is associated with primary treatment resistance and reduced overall survival in HGSC (10, 11). Patients whose tumors have CCNE1
Translational Relevance

High-grade serous ovarian cancer (HGSC) patients with Cyclin E1 (CCNE1) amplification represent a group with unmet clinical need, as they are unlikely to benefit from PARP inhibitors due to the mutual exclusivity of CCNE1 amplification and BRCA1/2 mutation (7, 12), and are less likely to respond to platinum agents.

In recent preclinical studies, we have shown a dependency on CDK2 (13) and HR activity (12) in CCNE1-amplified cell lines. Although targeted agents have been effective in the clinical setting across many cancers, the emergence of acquired resistance is common (14). Indeed, we reported in vitro resistance to CDK2 inhibitors through selection of a polyploid population in the CCNE1-amplified cell line OVCAR3 (13). Rational drug combinations are a potential strategy to prevent resistance (15), and may also facilitate improvements in the therapeutic window by reducing the doses of drugs required to achieve efficacy, resulting in fewer side effects (16). We therefore used a high-throughput drug screen to identify drug combinations that synergize with the CDK2 inhibitor dinaciclib (17) to selectively target CCNE1-amplified HGSC, and to overcome resistance in a cell line that has acquired resistance to CDK inhibitors in vitro (13). We identified several synergistic combinations, including dinaciclib and AKT inhibitors, and found that this synergy extended more generally to CCNE1-amplified HGSC cell lines. Our results suggest targeting CDK2 and the AKT pathway may be an important approach to the clinical management of CCNE1-amplified HGSC.

Materials and Methods

Ethics statement

All animal experiments were approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee and conducted in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Cell lines

Ovarian cancer cell lines were obtained from the National Cancer Institute Repository, actively passed for less than 6 months, and authenticated using short-tandem repeat markers to confirm their identity against the Cancer Genome Project database (Wellcome Trust Sanger Institute, Cambridge, United Kingdom) before use in experiments. Cells were maintained at 37°C and 5% CO2 (v/v), and cultured in RPMI1640 media containing 10% (v/v) FCS and 1% penicillin/streptomycin. Transfection and drug sensitivity assays were performed in the absence of antibiotics. Cell lines resistant to dinaciclib were generated utilizing methods as described previously (13). Briefly, OVCAR3 cells were plated in 6-well plates and treated with dinaciclib at the IC50 dose for two 72-hour periods (media removed and fresh drug added). Surviving cells were allowed to repopulate for 96 hours and the process repeated once. Remaining cells were cultured in media or in the presence of drug, and regularly monitored for sensitivity to dinaciclib. Six independent cell lines were generated in this fashion, and designated OVCAR3-RD1 to -RD6.

Short hairpin–mediated CDK2 knockdown

Short hairpin–mediated knockdown of CDK2 was performed by cloning CDK2-specific shRNA into a lentiviral tetracycline-inducible expression vector containing the optimized mIR-E backbone (18). The modified lentiviral vector pRRL-T3G-TurboGFP-miRE-PGK-mCherry-IREs-RTA3 (also referred to as LT3GEN) system includes a red (mCherry) fluorescent marker for transduction and a green (turboGFP) fluorescent marker for induction. Five CDK2-specific shRNA constructs were cloned into this system (see Supplementary Table S1 for sequences). For lentiviral production, HEK293T cells were transfected with plasmid DNA combined with the Lenti-X packaging system (Clontech Laboratories). Transfection, production of lentiviral particles, and transduction of target cells was performed as described by the manufacturer’s protocol. Doxycycline was used to induce shRNA expression, and transfection efficiency was validated by flow cytometry (FACS), and knockdown of individual hairpins by RT-PCR and Western blot analysis. The most efficient shRNA construct was taken forward for in vitro and in vivo experiments.

For in vivo experiments, xenograft tumors from transduced cells were generated as described below. Once tumors reached 100 mm3, mice were randomized into two groups to receive either normal food and water or doxycycline food and water (2 mg/mL in 2% sucrose) as a means of reliable induction of shRNA expression. Tumors were subsequently monitored as described below.

Cyclin E1 and AKT overexpression in Fallopian tube secretory epithelial cells

The immortalized fallopian tube secretory epithelial cell (FTSEC) line FT282 was obtained from Ronny Drapkin (University of Pennsylvania, Philadelphia, PA; ref. 19). Derivative cell lines were generated using pMSCV-mCherry-(empty) and pMSCV-mCherry-CCNE1, encoding full-length CCNE1. Additional cell lines were generated with pMSCV-GFP-myr-AKT1, pMSCV-GFP-myr-AKT2, and pMSCV-GFP-myr-AKT3, encoding the three different isoforms of myr-AKT (20). Plasmids were validated by sequencing, and expression of CCNE1, AKT1, AKT2, and AKT3 was validated by quantitative real-time PCR and Western blotting. Primer sequences are listed in Supplementary Table S2.

High-throughput compound screen

The compound library consisted of 73 targeted agents, 71 epigenetic agents, 208 kinase inhibitors, and 3,707 known drugs...
(21). All agents were dissolved in DMSO, and diluted to concentrations from 0.01 to 10 µmol/L. For targeted agents, epigenetic agents and kinase inhibitors, the primary screen was conducted using 1:1 concentrations; for the known drug library three concentrations were used. Compounds were dispensed into 384-well drug stock plates and stored at -20°C. Stock plates for dinaciclib at a fixed dose concentration (EC50) were prepared using a multichannel pipette before each assay.

Early passage cells were deposited into 384-well microtiter plates at 750–1,500 cells per well using a multidrop dispenser (Thermo Scientific) in 40 µL of media. Cells were allowed to adhere overnight. A MiniTrak IX (PerkinElmer Life Sciences) automated robotic platform was used to dispense compounds into assay plates. Compounds were added directly to assay plates using a 384, hydrophobic slotted pintoil (VP Scientific) calibrated to dispense 0.1 µL of DMSO compound solution. DMSO (0.1%) was used as negative control. Cells were exposed to drug for 48 hours, and cell viability measured using the CellTiter-Glo Luminescent Assay (Promega) and the EnVision Multilabel Plate Reader (PerkinElmer). Average viability was normalized to DMSO control wells, and EC50 dose was approximated by fitting a four-parameter dose–response curve using XLfit (IDBS).

**Xenograft studies**

Estrogen pellets were implanted subcutaneously into 4- to 6-week-old female NOD/SCID mice to facilitate the growth of xenografted cells. The pellet was implanted 3 days before injection of cells. Cell lines were grown in vitro, washed twice with PBS, and resuspended in 50% Matrigel (BD Biosciences) in PBS. Mice were injected subcutaneously with 5 x 105 cells in 100 µL and monitored at least twice weekly. Tumor volume was calculated using the equation: volume = (width)² x length/2. When tumors reached 100 to 150 mm³, mice were randomized into groups of five for treatment with vehicle alone or drug. Dinaciclib was prepared fresh before injection in 20% (v/v) hydroxypropyl-beta-cyclodextrin (Cyclodextrin Technologies Development, Inc.) and mice dosed twice weekly as a single agent via intraperitoneal injection. MK-2206 was reconstituted in 30% (v/v) Captisol (Ligand Technology) and dosed at 60 mg/kg three times per week as a single agent via oral gavage. For combination studies, MTDs of dinaciclib 20 mg/kg and MK-2206 60 mg/kg were dosed three times per week. All mice were monitored daily following drug dosing. Tumors were harvested at specific time points for biomarker analysis or at study endpoint, with half snap frozen in liquid nitrogen and half fixed in formalin and paraffin embedded for IHC. Percentage tumor growth inhibition (TGI) was calculated as 100 x (1-ΔT/ΔC) where ΔC and ΔT were determined by subtracting the mean tumor volume (in the vehicle control and treated groups, respectively) on day 1 of treatment, from the mean tumor volume on each day of assessment. Statistical analyses were performed using GraphPad Prism Version 6.0 (GraphPad) with ANOVA followed by Dunnett post hoc test to compare the tumor growth between treatment groups.

**CCNE1 and AKT status in primary ovarian tumor samples**

Genomic alterations identified in CCNE1 and genes involved in the PI3K–AKT–mTOR pathway were obtained from The Cancer Genome Atlas (TCGA) BioPortal (22, 23). All available data as of March 2015 were analyzed, comprising 316 primary ovarian serous cystadenocarcinoma samples (7).

**shRNA screen data**

Data from the Project Achilles was obtained to evaluate the interaction between CCNE1-amplified ovarian cancer cell lines and genes in the AKT pathway (24). Cell line copy number data were obtained from the Cancer Cell Line Encyclopedia (25). Only cell lines known to resemble HGSC according to their genomic characteristics (26) were used in the analysis (N = 14, see Supplementary Table S3). Cell lines with a log2 copy number ratio > 0.3 over the CCNE1 locus were designated as amplified (n = 9) and cell lines with a log2 copy number ratio < 0 were designated as unamplified (n = 5). Cell lines with CCNE1 gene expression greater than the median + 1 SD (n = 9) were defined as CCNE1-high expression, whereas cell lines with CCNE1 gene expression less than median (n = 5) were defined as CCNE1-low expression.

Additional methods for gene suppression studies, Western blot analysis, IHC, flow cytometry and drug sensitivity, clonogenic, proliferation, and anchorage-independent growth assays can be found in Supplementary Methods.

**Results**

CCNE1-amplified HGSC cells are selectively sensitive to CDK2 knockdown

We previously demonstrated in a limited number of cell lines that CCNE1-amplified HGSC cell lines are selectively sensitive to...
Figure 2.
CDK inhibitor dinaciclib results in modest tumor growth inhibition in vivo but is not synergistic in combination with bortezomib in vitro. **A**, Mean IC_{50} values for a panel of HGSC cell lines treated with dinaciclib generated from dose–response curves following standard MTS cell proliferation assays. Error bars, SEM, n = 3 experiments. **B**, In vivo effects of dinaciclib. Immunocompromised mice bearing OVCAR3 (CCNE1-amplified) or CAOV3 (CCNE1-unamplified) tumor xenografts were treated with vehicle or drug as described in Materials and Methods. Plots represent mean tumor volume change from baseline ± SEM, n = 5 mice per group. **C**, The percentage tumor growth inhibition following 21 days of treatment with vehicle or dinaciclib. Bars represent mean ± SEM, n = 5 mice per group. Statistical analysis performed with ANOVA followed by Dunnett post hoc test to compare the percentage tumor growth inhibition between the treatment groups. **D**, P < 0.01. **E**, Immunohistochemical analysis of Ki67 expression in OVCAR3 and CAOV3 tumor xenografts harvested 24 hours after dose of vehicle or dinaciclib. **F**, Formal assessment of synergy between dinaciclib and bortezomib using Chou–Talalay Isobologram analysis. Figures are generated with CalcuSyn 2.0. Data are normalized, with connecting line at X and Y corresponding to combination index = 1, representing line of additivity. Data points above the line are antagonistic, along or near the line are additive and points below the line are synergistic. **G–H**, Scatter plots showing EC_{50} values for library compounds in combination with dinaciclib from primary screen for the comparison between CCNE1-amplified and unamplified (**G**) and resistant versus parental (**H**). Data points in red represent compounds taken forward for secondary screen.
Figure 3.
Dinaciclib in combination with nonselective BH3 mimetics are synergistic in CDK inhibitor–resistant cell lines. Combination indexes for parental and CDK inhibitor–resistant cell lines tested against dinaciclib in combination with ABT-737 (A), ABT-263 (B), ABT-199 (C). Values represent mean ± SEM, n = 3. D, Western blot analysis demonstrating protein expression of Bcl-XL, Mcl-1, and PARP cleavage products in OVCAR3 parental and CDK inhibitor–resistant cell lines after treatment with dinaciclib and ABT-737. E, Expression of antiapoptotic proteins as assessed by quantitative real-time PCR. R-lines signify cell lines resistant to PHA53533. RD lines signify cell lines resistant to dinaciclib. Bars represent mean ± SEM, n = 3.
Dinaciclib in combination with two AKT inhibitors are synergistic in vitro and in vivo models of CCNE1-amplified HGSC. Combination indexes for a panel of HGSC cell lines tested against dinaciclib in combination with MK-2206 (A) and GSK2110183 (B). Values represent mean ± SEM. (Continued on the following page.)
Targeting CCNE1-Amplified Cancer by CDK2 and AKT Inhibition

CCNE1 and CDK2 knockdown mediated by siRNA (13). Following a recent analysis of ovarian cancer cell lines (26), we extended our analysis to a wider number of HGSC cell lines and confirmed consistent amplicon-dependent sensitivity to siRNA-mediated CCNE1 and CDK2 knockdown (Fig. 1A and Supplementary Fig. S1A and S1B). The OVCAR8 cell line has a low-level gain of CCNE1 and was not sensitive to CCNE1 or CDK2 knockdown (Fig. 1A). However, OVCAR8 does not overexpress cyclin E1 at the mRNA or protein level (Supplementary Fig. S2A) compared with other cell lines such as OVCAR4 that have similar CCNE1 copy number. These findings suggest a threshold of CCNE1/CDK2 dependency that may be relevant to patient selection in clinical trials targeting this oncogene in HGSC.

To validate the effect of CDK2 knockdown, we utilized a tetracycline-inducible shRNA targeting CDK2 (Fig. 1B). Consistent with the siRNA data, induction of CDK2 by shRNA resulted in reduced clonogenic survival, more evident in the CAOV3 cell line (Fig. 2B). The difference in amplicon-dependent sensitivity between gene suppression and pharmacologic inhibition may be due to the broad activity of dinaciclib, which, in addition to inhibiting CDK2, is also active against CDK1, 5, 9, and 12 (17, 27).

In addition to CDK2 inhibitors, we previously identified use of bortezomib, a proteasome inhibitor, as a potential therapeutic strategy for CCNE1-amplified HGSC (12). Although we did not observe amplicon-dependent sensitivity to dinaciclib, we investigated the interaction between dinaciclib and bortezomib to see whether the two drugs would be synergistic in combination. Using the Chou-Talalay methodology for drug combination studies (28), we did not observe a synergistic interaction with dinaciclib and bortezomib (Fig. 2E and F) in a panel of CCNE1-amplified and CCNE1-unamplified HGSC cell lines. Given this lack of synergism, we sought to identify selective synergistic drug combinations by adopting an unbiased high-throughput screening approach.

A high-throughput compound screen identifies synergistic drug combinations

We performed a high-throughput compound screen to identify combinations that would be synergistic in CCNE1-amplified cells, as well as combinations that would be selective in a CDK inhibitor–resistant cell line OVCAR3-R1-533533 (13). In the primary screen, 4,059 compounds (including duplicates) were combined with a fixed dose of dinaciclib as described in Materials and Methods. Dose–response curves were generated and manually curated, and compounds where a curve could not be fitted were excluded from the analysis. A full list of EC₅₀ values for each cell line and compound is given in Supplementary Tables S4 and S5. EC₅₀ values from the primary screen were used to make two pair-wise comparisons (Fig. 2G and H): (i) dinaciclib plus library compound comparing OVCAR3 (CCNE1-amplified) versus SKOV3 (CCNE1-unamplified) and (ii) dinaciclib plus library compound comparing OVCAR3 (parental) and OVCAR3-R1 (CDK inhibitor resistant). At the time of undertaking the screen, SKOV3 was a commonly used ovarian cancer cell line; however, recent studies have demonstrated that SKOV3 is unlikely to resemble HGSC (26). Therefore, any potential hits identified in the screen were subsequently validated using only HGSC cell lines.

Library compounds where the ratio of EC₅₀ was less than 0.5 were selected as hits for a secondary screen involving a total of 64 compounds (Supplementary Table S6 and S7). Compounds that...
appeared to have an additive effect with dinaciclib were selected as hits from the secondary screen and carried forward for further testing.

The final part of the screen involved assessing the level of synergy between the library compound hits and dinaciclib involving an 11-point titration of each compound. Using the Chou–Talalay methodology of constant-ratio drug combinations, a series of combination indexes were generated to identify synergistic interactions.

In the OVCAR3 parental cell line, there were no synergistic combinations identified between dinaciclib and the library compounds (Supplementary Table S8). In the OVCAR3-R1 cell line, there were a number of synergistic interactions identified (Supplementary Table S8). Nonselective BH3-mimetic agents ABT-263 and ABT-737 were synergistic in combination with dinaciclib, suggestive of a class effect. This was validated further in an independently derived dinaciclib-resistant cell line, OVCAR3-RD6 (Fig. 3A–B and Supplementary Fig. S4A–S4C). There was no synergistic interaction noted in the combination between dinaciclib and ABT-199 (Fig. 3C), a selective Bcl-2 antagonist. The combination of dinaciclib and ABT-737 resulted in a dose-dependent increase in apoptosis, observed only in CDK inhibitor–resistant cell lines as demonstrated by increase in PARP cleavage products on Western blot analysis (Fig. 3D). Mcl-1 protein expression was not observed in the OVCAR3-RD cell line resistant to dinaciclib (Fig. 3D). Real-time PCR demonstrated upregulation of antiapoptotic genes in the dinaciclib and PHA533533-resistant cell lines (Fig. 3E), but downregulation of MCL1 in the dinaciclib-resistant OVCAR3-RD cell lines. Dinaciclib is reported to have a greater effect on CDK9 compared with PHA533533 (13). Given that MCL1 is regulated by CDK9 activity (29), this may explain the reduction of MCL1 levels in the presence of dinaciclib. However, it is unclear why reduced MCL1 expression is also apparent in OVCAR3-RD cell lines even when grown in the absence of dinaciclib.

MK-2206, a pan-AKT inhibitor, was identified as a synergistic drug combination in the CDK inhibitor–resistant cell line, OVCAR3-R1. In validating this interaction between dinaciclib and MK-2206, we observed that this combination was also synergistic in CCNE1-amplified cell lines FUOV1 and parental OVCAR3 (Fig. 4A). This effect was similarly observed with another AKT inhibitor, GSK-2110183 (Fig. 4B), that was not included in the original high-throughput screen library. Exposure to dinaciclib and MK2206 resulted in significantly higher number of apoptotic cells, indicated by percentage of Annexin V–positive cells measured by FACS (Fig. 4C). This result was similarly observed on Western blot analysis, with appearance of PARP cleavage products following treatment of OVCAR3 cells with the combination of dinaciclib and MK-2206 (Supplementary Fig. S4D). As dinaciclib targets several CDKs in addition to CDK2 (17), we used siRNA knockdown of CDK2, CDK1, or CDK9 to determine the specificity of the synergistic effect of dinaciclib and MK-2206. We found that the synergy observed was predominantly mediated through CDK2 (Supplementary Fig. S4E).
Figure 6.
Cyclin E1 and AKT overexpression cooperates to promote uncontrolled growth in FTSECs. A, Western blot analysis of fallopian tube secretory cells transduced with cyclin E1, empty vector, and AKT1, AKT2, and AKT3 overexpression constructs. Blots are representative of three independently performed experiments. B, Proliferation assay of fallopian tube secretory cells (FT282) transduced with empty vector (EV), cyclin E1 (CCNE1), AKT2, and both cyclin E1 and AKT2 (CCNE1+AKT2). Plots represent mean of three independently performed experiments, error bars represent SEM. C, Clonogenic survival assay of FT282 cells transduced as labeled. Images (left) show cells fixed and stained with crystal violet. Bar chart represents mean of three independently performed experiments, error bars represent SEM. Statistical significance (t test) calculated by comparison with FT282 cells transduced with cyclin E1 (FT282-CCNE1). D, Anchorage-independent assay of FT282 cells transduced as labeled. Images (left) represent cells fixed with 2% paraformaldehyde and captured using an Olympus IX81 live cell imager. Bar chart represents mean of three independently performed experiments, error bars represent SEM. Statistical significance (t test) calculated by comparison with FT282 cells transduced with cyclin E1 (FT282-CCNE1); *, P < 0.05; **, P < 0.01.
Dinaciclib and MK-2206 are selectively synergistic in CCNE1-amplified cell lines in vivo

The in vivo effect of dinaciclib and MK-2206 was assessed using xenograft models from CCNE1-amplified and unamplified cell lines, OVCAR3 and CAOV3, respectively. The combination was significantly more effective than each single agent alone in the CCNE1-amplified model (Fig. 4D and E), whereas there was no statistically significant effect of the combination compared to single-agent treatment in the CCNE1-unamplified model. After a treatment period of three weeks with dinaciclib and MK-2206, xenograft tumors began regrowing within 10 days of treatment cessation. Rechallenge with the same drug combination resulted in significant tumor regression (Supplementary Fig. S4F), indicating continued sensitivity to the combination. Consistent with this effect on tumor growth, treatment with dinaciclib and MK-2206 resulted in inhibition of cell proliferation and induction of apoptosis, as assessed by Ki67 and cleaved caspase-3 IHC on tumors harvested at 24 hours (Fig. 4F and G). Taken together, the high-throughput screen identified a novel combination of dinaciclib and MK-2206 that appeared to be selectively synergistic in CCNE1-amplified HGSC cell lines both in vitro and in vivo.

CCNE1 and AKT2 are frequently coamplified in primary HGSC samples

We sought to investigate whether there was evidence for an interaction between CCNE1 amplification and the AKT pathway in primary tumor samples. Analysis of TCGA dataset indicated that CCNE1 and AKT2 amplification events cooccur (P < 0.001; Supplementary Fig. S5). This observation was not seen with other isoforms of AKT or genes in the AKT pathway. To examine the relationship between CCNE1 amplification and the AKT pathway further, we made use of data from Project Achilles, a genome-wide shRNA screen of synthetic lethality in 216 cancer cell lines (24). The abundance of shRNA sequence relative to a reference pool was statistically significant in genes in the AKT pathway as a potential mechanism of genes in the AKT pathway as a potential mechanism of CCNE1 amplification and the AKT pathway (Fig. 5). AKT2, was observed, indicated by a depletion of shRNAs targeting CCNE1 copy number or expression. A statistically significant dependence on genes in the AKT pathway, including AKT2, was observed, indicated by a depletion of shRNAs targeting these genes in cell lines with CCNE1 amplification or overexpression (Fig. 5). CDK2 was included in the analysis as a control, and consistent with our previous analysis, was shown to be required in CCNE1-amplified cells (13).

Cyclin E1 and AKT overexpression cooperates to promote uncontrolled growth in FTSECs

Previously, Karst and colleagues demonstrated that cyclin E1 overexpression combined with TP53 mutation in FTSECs resulted in increased proliferation, colony-forming ability, and colony formation in soft agar (19). However, cyclin E1 overexpression alone did not result in complete transformation, suggesting that additional events are required.

We examined the interaction between cyclin E1 and AKT overexpression in FTSECs by overexpressing the myristoylated, active forms of AKT1, AKT2, and AKT3 (20). Expression of each AKT isoform and cyclin E1 was validated with Western blot analysis (Fig. 6A) and RT-PCR (Supplementary Fig. S6A). Overexpression of AKT isoforms led to increased expression of AKT downstream targets (Supplementary Fig. S6B). AKT2 and cyclin E1 overexpression alone or in combination showed a trend toward increased proliferation compared with empty vector alone (Fig. 6B), and AKT2 or AKT3 overexpression in combination with cyclin E1 showed a trend toward enhanced clonogenic colony formation in comparison with overexpression of cyclin E1 alone (Fig. 6C). There was a significant increase in soft agar colony formation with the overexpression of AKT2 or AKT3 in combination with cyclin E1 compared with overexpression of cyclin E1 alone (Fig. 6D). These findings support an interaction between cyclin E1 and AKT pathway to promote uncontrolled growth in FTSECs, and may explain synergism observed between dinaciclib and MK-2206 in CCNE1-amplified HGSC.

Discussion

HGSC patients with CCNE1 amplification have a clear unmet need in terms of effective therapies. In this study, we validate CDK2 as a selective target in CCNE1-amplified HGSC using shRNA-mediated gene suppression in vitro and in vivo. However, we did not observe similar amplicon-dependent specificity to dinaciclib, a small-molecule inhibitor targeting CDKs. This may be due to the nonspecificity of inhibitors such as dinaciclib or a role for kinase-independent activities of CCNE1 in amplified HGSC (30). Our findings highlight the potential differences between inhibition of kinase activity and complete suppression of CCNE1 or CDK2 gene expression.

In addition to CDK2, dinaciclib targets CDK1, 5, 9, and 12 (17, 27). CDK9 phosphorylates the carboxyl-terminal repeat domains of RNA polymerase II, and inhibition of CDK9 by dinaciclib results in rapid downregulation of mRNA transcripts and proteins with short half-lives such as the antiapoptotic BCL2 family member, Mcl1 (17). Preclinical studies have indicated dinaciclib-mediated targeting of Mcl-1 may be an effective therapeutic approach in a number of different cancers (17). Inhibition of CDK2 kinase activity may also differ significantly from complete suppression of gene expression, resulting in varying downstream and compensatory effects (31, 32). Studies with knockout experiments indicate that CDK2 functions appear redundant with CDK1, although in our studies, we did not observe upregulation of CDK1 expression following CDK2 knockdown in vitro or in vivo (data not shown).

Although we observed a difference in the amplicon-dependent sensitivity of CDK2 gene suppression compared with pharmacologic inhibition, dinaciclib remains a potent CDK2 inhibitor with single-agent activity in CCNE1-amplified HGSC cell lines and is one of the most clinically advanced CDK2 inhibitors (33). Therefore, to more effectively target CCNE1-amplified HGSCs, we performed a combinatorial drug screen to identify compounds that would synergize with dinaciclib. We also sought to identify compounds that may potentially overcome resistance to dinaciclib, a common occurrence in the clinical use of targeted small-molecule inhibitors, by testing a cell line that was resistant to CDK inhibitors. Dinaciclib in combination with MK-2206, an AKT inhibitor, was identified as a synergistic combination in targeting CDK inhibitor–resistant cell lines. This supported our previous work that identified increased AKT1 copy number and upregulation of genes in the AKT pathway as a potential mechanism of resistance to CDK2 inhibitors (13). In validating this finding, we observed selective, potent synergism between dinaciclib and MK-2206 in vitro and in vivo models of CCNE1-amplified HGSCs, including parental OVCAR3 cells. This interaction was not initially observed in the primary high-throughput screen. However,
the use of SKOV3 cell line as a comparator in the screen may be a potential confounder, as the selection of compounds as hits from the primary screen was based on a difference in the EC50 values between the two cell lines tested, OVCAR3 and SKOV3. Recently, multiple studies characterizing the genomic profile of commercially available ovarian cancer cell lines have shown that many of these cell lines, including SKOV3, may not accurately resemble HGSC (26, 34–36).

Synergy between dinaciclib and MK-2206, as well as another AKT-specific inhibitor GSK2110183, but an absence of a synergistic combination with other inhibitors of the PI3K–AKT–mTOR pathway suggests that the interaction with CCNE1 may be specific to AKT. Analysis of genomic data from patients demonstrated a significant cooccurrence of CCNE1 and AKT2 amplification, which may in part be explained by colocalization on chromosome 19q. However, FUOV1, which has CCNE1-amplification without AKT2-amplification (25), was equally sensitive to the combination of dinaciclib and AKT inhibitors. Coexpression of AKT2 or AKT3 with cyclin E1 in a TP53-mutant FTSECI cell line resulted in increased proliferation and anchorage-independent growth. Analysis of data from Project Achilles indicates that HGSC cell lines that have CCNE1 amplification or overexpression are dependent on multiple genes within the AKT pathway. We previously performed a pathway analysis of genes coexpressed with CCNE1 amplification and observed an enrichment of genes involved in AKT signaling (12). Collectively, these data suggest a specific dependency of CCNE1-amplified tumors for AKT activity.

Dinaciclib and MK-2206 have previously been shown to be active against pancreatic adenocarcinoma (37). In KRAS-mutant pancreatic cancer patient–derived xenografts, Hu and colleagues (37) demonstrated efficacy of dinaciclib combined with MK-2206. They proposed that sensitivity was due to the effect of dinaciclib on CDK5, and in turn, inhibition of RAL pathway. On the basis of these results, a phase 1 clinical trial (NIH Trial NCT01783171) of dinaciclib and MK-2206 has been initiated in patients with advanced pancreatic cancer. While this trial will provide safety and recommended dosing of the combination, patients are not preselected on the basis of tumor CCNE1 amplification, and the mechanism of interaction and biomarkers that predict response are likely to be different in pancreatic cancer compared with HGSC.

Other combinations were also identified from the high-throughput screen. In particular, nonselective BH3-mimetic compounds ABT-737 and ABT-263 were synergistic in combination with dinaciclib in CDK inhibitor–resistant cell lines. There was no synergistic interaction between dinaciclib and the Bcl-2–specific antagonist, ABT-199, indicating that the targeting of multiple antiapoptotic proteins is potentially required to overcome resistance to CDK2 inhibitors. This observation is supported by upregulation of multiple genes in this pathway including BCL-2, BCL-XL, and BCL-W in resistant cell lines. However, the use of ABT-737 or ABT-263 in combination with dinaciclib in vivo is hindered by significant toxicities, particularly hematologic (Joel Leverson, personal communication), and are therefore unlikely to have clinical utility.

Biomarker-driven trials in HGSC are needed to improve clinical outcomes. HGSC patients with CCNE1 amplification are a subset that requires different treatment approaches, given that they have HR-proficient tumors, and as such, are likely to have poor responses to platinum-based chemotherapy and PARP inhibitors. However, targeted therapies when used alone may not be sufficient to induce selective, cytotoxic effects, and often result in the development of resistance. Combination therapies may potentially be a strategy to overcome these limitations. High-throughput drug screening is an unbiased approach to identify novel therapeutic strategies, and we have identified dinaciclib and MK-2206 as a combination that may prove to selectively target patients with CCNE1-amplified HGSC. Further work incorporating additional clinically relevant models and novel combinations will inform the design of rational clinical trials targeting CCNE1-amplified HGSC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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References
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