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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.

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Experiment Design—To examine dependency on *CDK2* in *CCNE1* amplified HGSC, we utilised 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 co-amplification of *CCNE1* and *AKT2*. Over-expression 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.

Keywords

Ovarian cancer; Cyclin E1; CDK2 inhibitors; AKT inhibitors

Introduction

Targeted therapies have changed the management of many cancers types, resulting in significant improvements in clinical response rates and survival (1). However, although the anti-angiogenic monoclonal antibody bevacizumab (2, 3) and the poly(ADP-ribose) polymerase (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.

HGSC are characterised 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 HGSC, primarily associated with germline 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-characterised 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*-amplification represent a group with unmet clinical need, as they are unlikely to benefit from PARP inhibitors by virtue of the mutual exclusivity of *CCNE1*-amplification and *BRCA1/2* mutation (7, 12), and are less likely to respond to platinum agents.

In recent pre-clinical 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 synergise 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 that this synergy extended more generally to *CCNE1*-amplified HGSC 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 passaged for less than six months and authenticated using short-tandem repeat markers to confirm their identity against the Cancer Genome Project database (Wellcome Trust Sanger Institute, Cambridgeshire, United Kingdom) prior to use in experiments. Cells were maintained at 37°C and 5% CO₂ (vol/vol), and cultured in RPMI 1640 media containing 10% (vol/vol) FCS and 1% penicillin/streptomycin (P/S). Transfection and drug sensitivity assays were performed in the absence of antibiotics. Cell lines resistant to dinaciclib were generated utilising 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 LT3GECIR) 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 S2 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. 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 randomised into two groups to receive either normal food and water or doxycycline food and water (2mg/mL in 2% sucrose) as a means of reliable induction of shRNA expression. Tumors were subsequently monitored as described below.

Cyclin E1 and AKT over-expression in Fallopian tube secretory epithelial cells

The immortalised fallopian tube secretory epithelial cell (FTSEC) line FT282 was obtained from Ronny Drapkin (University of Pennsylvania) (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 S1.

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 μM to 10 μM. For targeted agents, epigenetic agents and kinase inhibitors, the primary screen was conducted using 11 concentrations; for the known drug library 3 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 (EC30) were prepared using a multichannel pipette prior to each assay.

Early passage cells were deposited into 384-well microtiter plates at 750–1,500 cells per well using a multidrop dispenser (Thermo) in 40μL of media. Cells were allowed to adhere overnight. A MiniTrak™ IX (Perkin Elmer 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 pintool (VP Scientific) calibrated to dispense 0.1μL of DMSO compound solution. 0.1% DMSO was used as negative control. Cells were exposed to drug for 48 hours, and cell viability measured using the CellTitre-Glo luminescent assay (Promega) and the EnVision multilabel plate reader (PerkinElmer). Average viability was normalised 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 4–6 week old female NOD/SCID mice to facilitate the growth of xenografted cells. The pellet was implanted 3 days prior to 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 5×10^6 cells in 100 μ L, and monitored at least twice weekly. Tumor volume was calculated using the equation: Volume = (width)² $\times$ length/2. When tumors reached 100–150 mm³, mice were randomised into groups of five for treatment with vehicle alone or drug. Dinaciclib was prepared fresh prior to injection in 20% (w/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% (w/v) Captisol (Ligand Technology) and dosed at 60 mg/kg three times per week as a single agent via oral gavage. For combination studies, maximum tolerated doses of dinaciclib 20mg/kg and MK-2206 60mg/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 immunohistochemistry. Percentage tumor growth inhibition (TGI) was calculated as $100 \times (1 - T/C)$ where C and T 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, La Jolla, CA, USA) with analysis of variance (ANOVA) followed by Dunnett's 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) cBioPortal (22, 23). All available data as at March 2015 was analysed, 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 high grade serous ovarian cancer 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, immunohistochemistry, 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 *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, Supplementary Fig S1A–B). 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 over-express cyclin E1 at the mRNA or protein level (Supplementary Fig S1B–C), compared to 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 utilised a tetracycline-inducible shRNA targeting *CDK2* (Fig 1B). Consistent with the siRNA data, inhibition of *CDK2* by shRNA resulted in reduced clonogenic survival, more evident in the *CCNE1*-amplified cell line, OVCAR3 compared to the *CCNE1*-unamplified cell line CAOV3 (Fig 1C). Knockdown of *CDK2* was validated at the protein level (Supplementary Figure S2A). Cell-cycle analysis demonstrated arrest in G1, seen only in the OVCAR3 cell line (Fig 1D). We did not observe significant levels of apoptosis following *CDK2* knockdown, as assessed by percentage of Annexin V positive cells measured by FACS (Supplementary Figure S2B).

Cells transduced with *CDK2*-shRNA were grown as xenografts in nod/scid mice to examine the effects of *CDK2* knockdown *in vivo*. Consistent with the *in vitro* data, attenuation of *CDK2* expression in the OVCAR3 xenograft model resulted in significant tumor growth arrest in the group receiving doxycycline in food and water compared to controls. (Figure 1E–F). Induction of shRNA by doxycycline was monitored by *CDK2* gene expression measured by RT-PCR (Supplementary Figure S2C). Reduced Rb1 phosphorylation was observed following *CDK2* knockdown in OVCAR3 tumors harvested at seven days following induction (Fig 1G), providing a biomarker of targeting cyclinE1/*CDK2*.

Taken together, *CCNE1*-amplified HGSC appear selectively sensitive to siRNA- and shRNA-mediated knockdown of *CDK2* both *in vitro* and *in vivo*. These findings support our previous studies and point to *CDK2* as a potential therapeutic target in *CCNE1*-amplified HGSC.

CDK2 inhibitor dinaciclib delayed tumor growth in CCNE1-amplified HGSC xenografts

Consistent with siRNA data, we previously showed in a limited number of cell lines selective sensitivity of *CCNE1*-amplified cell lines to dinaciclib, a potent *CDK2* inhibitor in advanced clinical development (13). However, in this study, when tested across a broader panel of HGSC cell lines, there did not appear to be a clear amplicon dependent sensitivity (Fig 2A), in contrast to the siRNA and shRNA data. Furthermore, activity *in vivo* was also seen in a xenograft model developed from a *CCNE1*-unamplified cell line, CAOV3 (Fig 2A–D). The difference in amplicon dependent sensitivity between gene suppression and

pharmacological 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–2F) 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 found in Supplementary Table S4–S5.

EC50 values from the primary screen were used to make two pair-wise comparisons (Fig 2G–2H):

1) dinaciclib plus library compound comparing OVCAR3 (*CCNE1*-amplified) versus SKOV3 (*CCNE1*-unamplified) and 2) 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–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). Non-selective 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, Supplementary Fig S4A–C). 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-RD6 cell line resistant to dinaciclib (Fig 3D). Real-time PCR demonstrated up-regulation of anti-apoptotic genes in the dinaciclib and PHA533533-resistant cell lines (Fig 3E), but down-regulation of *MCL1* in the dinaciclib resistant OVCAR3-RD cell lines. Dinaciclib is reported to have a greater effect on CDK9 compared to 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 in *CCNE1*-amplified cell lines, 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 Figure 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).

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–E), whereas there was no statistically significant effect of the combination compared to single agent treatment in the *CCNE1*-unamplified model. Following a treatment period of three weeks with dinaciclib and MK-2206, xenograft tumors began regrowing within 10 days of treatment cessation. Re-challenge 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 immunohistochemistry on tumors harvested at 24 hours (Fig 4F–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 co-amplified in primary HGSC samples**

We sought to investigate whether there was evidence for an interaction between these *CCNE1*-amplification and the AKT pathway in primary tumor samples. Analysis of TCGA dataset indicated that *CCNE1* and *AKT2* amplification events co-occur ($p < 0.001$, 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 measured by microarray to identify genes essential for survival. We analysed the effect of shRNA targeting genes within the AKT pathway, restricting the analysis to HGSC cell lines, classified according to *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 over-expression co-operates to promote uncontrolled growth in FTSEC

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

We examined the interaction between cyclin E1 and AKT over-expression in FTSEC 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 (Fig 6A) and RT-PCR (Supplementary Fig S6A). Over-expression of AKT isoforms led to increased expression of AKT downstream targets (Supplementary Fig S6B). AKT2 and cyclin E1 over-expression alone or in combination showed a trend towards increased proliferation compared to empty vector alone (Fig 6B), and AKT2 or AKT3 over-expression in combination with cyclin E1 showed a trend towards enhanced clonogenic colony formation in comparison to over-expression of cyclin E1 alone (Fig 6C). There was a significant increase in soft agar colony formation with the over-expression of AKT2 or AKT3 in combination with cyclin E1 compared to over-expression of cyclin E1 alone (Fig 6D). These findings support an interaction between cyclin E1 and AKT pathway to promote uncontrolled growth in FTSEC, 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 utilising 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 non-specificity of inhibitors such as dinaciclib or a

role for kinase independent activities of *CCNE1* in amplified HGCS (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 down-regulation of mRNA transcripts and proteins with short half-lives such as the anti-apoptotic BCL2 family member, Mcl1 (17). Pre-clinical 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 up-regulation 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 to pharmacological 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, in order to more effectively target *CCNE1*-amplified HGSC, we performed a combinatorial drug screen to identify compounds that would synergise 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 up-regulation 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 HGSC, 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 characterising 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).

Synergism 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 co-occurrence of *CCNE1* and *AKT2*-amplification, which may in part be explained by co-localisation on chromosome 19q. However, FUOV1, which has *CCNE1*-amplification without *AKT2*-amplification (25), was equally sensitive to the combination of dinaciclib and AKT inhibitors. Co-expression of AKT2 or AKT3 with cyclin E1 in a *TP53*-mutant FTSEC 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 over-expression are dependent on multiple genes within the AKT pathway. We previously performed a pathway analysis of genes co-expressed with *CCNE1*-amplification and observed an enrichment of genes involved in AKT signalling (12). Collectively, these data suggests 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 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. Based on 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 tumoral *CCNE1*-amplification, and the mechanism of interaction and biomarkers that predict response are likely to be different in pancreatic cancer compared to HGSC.

Other combinations were also identified from the high throughput screen. In particular, non-selective 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 anti-apoptotic proteins is potentially required to overcome resistance to CDK2 inhibitors. This observation is supported by up-regulation 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 haematological (Joel Levenson, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

High grade serous ovarian cancer (HGSC) patients with Cyclin E1 (*CCNE1*) amplification represent a group with high unmet clinical need. Novel therapies are needed to improve outcomes in these patients, given that *CCNE1* amplified tumors are unlikely to respond to chemotherapy or PARP inhibitors, and are associated with poor overall survival. Here, we validate *CDK2* as a selective target for *CCNE1* amplified cell lines. We performed a high throughput compound screen and identified a number of potential therapeutic combinations. We focused on dinaciclib and AKT inhibitors, and demonstrate selective and potent activity in *CCNE1* amplified HGSC. We further show co-operation between *CCNE1* and *AKT*, both in genomic data from TCGA and functionally in fallopian tube secretory cells. This study demonstrates approaches to targeting an important subset of solid cancers, and for the first time provides evidence to support the design of a rational clinical trial that targets *CCNE1* amplified HGSC.

Figure 1. CDK2 knockdown via siRNA and shRNA *in vitro* and *in vivo* results in selective reduction in clonogenic survival and tumor growth arrest in *CCNE1*-amplified HGSC

(A) Clonogenic survival after transfection with *CCNE1* and *CDK2* siRNAs in panel of HGSC cell lines. Average percentage of discrete colonies formed after 7–10 days relative to no siRNA controls shown (n=3). Error bars indicate SEM. Statistical significance (*t* test) calculated by comparison with non-silencing (NS) siRNA in the same cell line. **, *p*<0.01, ***, *p*<0.001, ****, *p*<0.0001.

(B) Schematic of conditional LT3GECIR lentiviral vector showing inducible transcripts produced by vector.

(C) Clonogenic survival after induction of a non-specific or CDK2 targeting shRNA in OVCAR3 (*CCNE1*-amplified) and CAOV3 (*CCNE1*-unamplified). Average percentage of discrete colonies formed after 7–10 days relative to no induction shown (n=3). Statistical significance (*t* test) calculated by comparison with non-induced (–Dox) in the same cell line. **, *p*<0.01, ***, *p*<0.001.

(D) Cell cycle analysis following CDK2 knockdown with inducible shRNA. Proportion of cells in G1, S or G2 phase for PI stained cells analysed by flow cytometry 72 hours after induction with doxycycline. Mean of 3 independently performed experiments shown. Statistical significance (*t* test) calculated by comparison with non-induced (–Dox) in the same cell line. ***, *p*<0.001.

(E) Mean percentage change in tumor volume ± SEM following induction of a non-specific (NS) or CDK2 (sh6) shRNA in subcutaneous xenograft tumors grown in immunocompromised mice, generated from OVCAR3 and CAOV3. Induced and non-

induced groups as marked, n=5 per group. **, p<0.001, unpaired t-test comparison of mean percentage tumor volume change.

(F) Percentage tumor growth inhibition following induction of non-specific (NS) or CDK2 (sh6) shRNA with doxycycline. Bars represent mean $\pm$ SEM, n=5 mice per group. Statistical analysis performed with analysis of variance (ANOVA) followed by Dunnett's post hoc test to compare the percentage tumor growth inhibition between the treatment groups. ****, p<0.0001.

(G) Immunohistochemistry assessment of phospho-Rb with or without doxycycline treatment in OVCAR3 xenograft tumor.

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 IC₅₀ values for a panel of HGSC cell lines treated with dinaciclib generated from dose-response curves following standard MTS cell proliferation assays. Error bars represent SEM, n=3 experiments.

(B) *In vivo* effects of dinaciclib. Immunocompromised mice bearing OVCAR3 (*CCNE1*-amplified) or CAO3 (*CCNE1*-unamplified) tumor xenografts were treated with vehicle or drug as described in methods. Plots represent mean tumor volume change from baseline $\pm$ SEM, n=5 mice per group.

(C) Percentage tumor growth inhibition following 21 days of treatment with vehicle or dinaciclib. Bars represent mean $\pm$ SEM, n=5 mice per group. Statistical analysis performed with analysis of variance (ANOVA) followed by Dunnett's post hoc test to compare the percentage tumor growth inhibition between the treatment groups. **, p<0.01.

- (D) Immunohistochemistry analysis of Ki67 expression in OVCAR3 and CAOV3 tumor xenograft harvested 24 hours after dose of vehicle or dinaciclib.
- (E) Formal assessment of synergy between dinaciclib and bortezomib using Chou-Talalay Isobologram analysis. Figures are generated with CalcuSyn 2.0. Data are normalised, with connecting line at X and Y corresponding to combination index = 1, representing line of additivity. Datapoints above the line are antagonistic, along or near the line are additive and points below the line are synergistic.
- (F) Combination indexes for a panel of HGSC cell lines tested against dinaciclib in combination with bortezomib. Values represent mean $\pm$ SEM, n=3.
- (G–H) Scatter plots showing EC₅₀ values for library compounds in combination with dinaciclib from primary screen for the comparison between (G) *CCNE1*-amplified and unamplified; and (H) resistant versus parental. Data points in red represent compoundstaken forward for secondary screen.

Figure 3. Dinaciclib in combination with non-selective 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 (A) ABT-737, (B) ABT-263, (C) ABT-199. Values represent mean $\pm$ SEM, n=3.

(D) Western blot 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 anti-apoptotic proteins as assessed by quantitative real-time PCR. R-lines signify cell lines resistant to PHA533533. RD-lines signify cell lines resistant to dinaciclib. Bars represent mean $\pm$ SEM, n=3.

Figure 4. 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 (A) MK-2206 and (B) GSK2110183. Values represent mean $\pm$ SEM, n=3.

(C) HGSC cell lines were cultured *in vitro* with dinaciclib, MK-2206 or the combination for 24 hours and then analysed using flow cytometry for Annexin V/propidium iodide positivity. Bars represent mean $\pm$ SEM, n=3. *, p<0.05; **, p<0.01; ***, p<0.001; unpaired t-test.

(D) *In vivo* effects of vehicle, dinaciclib, MK-2206 or combination. Immunocompromised mice bearing OVCAR3 (CCNE1-amplified) or CAOV3 (CCNE1-unamplified) tumor xenografts were treated with vehicle or drug as described in methods. Plots represent mean tumor volume change from baseline $\pm$ SEM, n=5 mice per group.

(E) Percentage tumor growth inhibition following 21 days of treatment with vehicle, dinaciclib, MK-2206 or the combination. Bars represent mean $\pm$ SEM, n=5 mice per group. Statistical analysis performed with analysis of variance (ANOVA) followed by Dunnett's post hoc test to compare the percentage tumor growth inhibition between the treatment groups. *, p<0.05; **, p<0.01; ***, p<0.0001.

(F) Quantitation of immunohistochemistry staining for Ki67 and cleaved caspase-3. Bars represent mean percentage of Ki67 or cleaved caspase 3 positive cells relative to background number of cells measured $\pm$ SEM, n=3 in each group. Statistical analysis performed by analysis of variance (ANOVA) with Tukey's multiple comparison test to compare between treatment groups.

(G) Subcutaneous tumors were obtained after 24hrs of treatment and were examined by immunohistochemistry for biomarker analysis. Rb phosphorylation was inhibited by dinaciclib, but not MK-2206 treatment. AKT phosphorylation was inhibited by MK-2206,

but not dinaciclib treatment. Proliferation (Ki67) was inhibited and apoptosis (cleaved caspase-3) was induced by the combination of dinaciclib and MK-2206 in *CCNE1*-amplified xenograft model (OVCAR3).

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Figure 5. *CCNE1* and *AKT2* are co-amplified in primary HGSC samples

Dot plots of median shRNA abundance for each gene targeted by shRNA in HGSC cell lines, stratified by *CCNE1* copy number or expression. Depletion of shRNA abundance within a group suggests requirement for maintained expression of its target gene. Only genes with a statistically significant difference shown, see Supplementary Table S3 for list of genes and cell lines analysed. Statistical significance (*t* test) calculated by comparison between *CCNE1*-amplified and unamplified or *CCNE1* over-expressing and low expressing cell lines. *, $p < 0.05$, **, $p < 0.01$.

Figure 6. Cyclin E1 and AKT over-expression co-operates to promote uncontrolled growth in fallopian tube secretory epithelial cells

(A) Western Blot analysis of fallopian tube secretory cells transduced with cyclin E1, empty vector and AKT1, AKT2 and AKT3 over-expression 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 3 independently performed experiments, error bars represent SEM.

(C) Clonogenic survival assay of FT282 cells transduced as labelled. Images (left) show cells fixed and stained with crystal violet. Bar chart represents mean of 3 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 labelled. Images (left) represent cells fixed with 2% paraformaldehyde and captured using an Olympus IX81 live cell imager. Bar chart represents mean of 3 independently performed experiments, error bars

represent SEM. Statistical significance (t test) calculated by comparison with FT282 cells transduced with cyclin E1 (FT282-CCNE1). *, $p < 0.05$, **, $p < 0.01$.

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