Dual CCNE1/PIK3CA targeting is synergistic in CCNE1-amplified/PIK3CA-mutated uterine serous carcinomas in vitro and in vivo

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Background: Clinical options for patients harbouring advanced/recurrent uterine serous carcinoma (USC), an aggressive variant of endometrial tumour, are very limited. Next-generation sequencing (NGS) data recently demonstrated that cyclin E1 (CCNE1) gene amplification and pik3ca driver mutations are common in USC and may therefore represent ideal therapeutic targets.

Methods: Cyclin E1 expression was evaluated by immunohistochemistry (IHC) on 95 USCs. The efficacy of the cyclin-dependent kinase 2/9 inhibitor CYC065 was assessed on multiple primary USC cell lines with or without CCNE1 amplification. Cell-cycle analyses and knockdown experiments were performed to assess CYC065 targeting specificity. Finally, the in vitro and in vivo activity of CYC065, Taselisib (a PIK3CA inhibitor) and their combinations was tested on USC xenografts derived from CCNE1-amplified/pik3ca-mutated USCs.

Results: We found that 89.5% of the USCs expressed CCNE1. CYC065 blocked cells in the G1 phase of the cell cycle and inhibited cell growth specifically in CCNE1-overexpressing USCs. Cyclin E1 knockdown conferred increased resistance to CYC065, whereas CYC065 treatment of xenografts derived from CCNE1-amplified USCs significantly reduced tumour growth. The combination of CYC065 and Taselisib demonstrated synergistic effect in vitro and was significantly more effective than single-agent treatment in decreasing tumour growth in xenografts of CCNE1-amplified/pik3ca-mutated USCs.

Conclusions: Dual CCNE1/PIK3CA blockade may represent a novel therapeutic option for USC patients harbouring recurrent CCNE1-amplified/pik3ca-mutated tumours.

Keywords: uterine serous carcinoma; CCNE1; PIK3CA; CDK2; CDK9

Uterine serous carcinoma (USC) constitutes only 10% of all endometrial cancers, but accounts for a disproportionate number of deaths among women affected with endometrial tumours (Siegel et al., 2015). Currently, the clinical management of advanced-stage USC includes cytoreductive surgery followed by systemic cytotoxic chemotherapy with or without localised radiation therapy (Hendrickson et al., 1982; Moore and Fader, 2011). Unfortunately, secondary to the intrinsic and/or rapid development of resistance to chemotherapy most advanced stage USC patients develop recurrent disease, which is fatal in the majority of the cases (Goff et al., 1994). Clinical options for the treatment of recurrent chemotherapy-resistant disease are very limited (Schwartz, 2006).
Novel, more effective treatment modalities for the management of recurrent USC are desperately needed. The genomic landscape of endometrial carcinoma has recently been evaluated by the Cancer Genome Atlas (TCGA) Research Network using multiple sequencing-based technologies (Cancer Genome Atlas Research Network et al., 2013). Data revealed that serous and serous-like endometrioid tumours have a unique genomic profile among endometrial cancers, characterised by frequent TP53 mutations and extensive copy number alterations (Cancer Genome Atlas Research Network et al., 2013). Importantly, the TCGA data also showed that ~85% of these tumours exhibited a significant deregulation in the expression of cell-cycle-related genes (Cancer Genome Atlas Research Network et al., 2013). Consistent with these results, a comprehensive next-generation sequencing (NGS) evaluation of 57 USCs from our research group has recently shown that alterations in the cell-cycle pathway occurred in up to 88% of these tumours (Zhao et al., 2013). Remarkably, the amplification of the gene encoding for cyclin E1 (CCNE1) was reported in 48% of the samples analysed.

Cyclin E1 is a critical regulator of the G1–S phase transition in mammalian cells. Following binding to cyclin-dependent kinase 2 (CDK2), the CCNE1/CDK2 kinase complex is responsible for the regulation of several important S-phase processes including retinoblastoma (Rb) phosphorylation, histone biosynthesis and prereplication complex assembly (Reed, 1996; Ekholm and Reed, 2000; Hubalek et al., 2004). Upregulation of CCNE1 has been reported in many types of human tumours including high-grade serous ovarian and breast cancers and has been shown to correlate with worse prognosis (Sui et al., 2001; Rosen et al., 2006; Scalltriti et al., 2011; Lundgren et al., 2015). Although these evidences identify CCNE1 as an attractive therapeutic target in multiple tumour types, developing molecules to specitically target CCNE1 have so far been difficult because CCNE1 operates as a regulatory subunit of CDK2 rather than as a kinase or a receptor. The use of CDK2 inhibitors to indirectly interfere with CCNE1 function has been therefore proposed as an alternative approach in the past years (Asghar et al., 2015; Taylor-Harding et al., 2015; Yang et al., 2015). Accordingly, the antitumour activity of CYC065 (Cyclacel Ltd, Dundee, UK), a second-generation, orally available ATP-competitive inhibitor of CDK2/9 kinases has been previously evaluated on CCNE1-amplified breast cancer cells (Scalltriti et al., 2011). Results showed that CYC065 is able to significantly inhibit tumour growth in xenografts of CCNE1-amplified breast cancer. Importantly, this study identified CCNE1 amplification as one of the acquired mechanism of resistance to Trastuzumab, a monoclonal antibody targeting Her2/neu, and demonstrated that the treatment of Trastuzumab-resistant/CCNE1-amplified tumour cells with CYC065 was able to overcome such resistance, suggesting a molecular connection between CCNE1 and the Her2/P13K/ Akt/mTOR signalling pathways. Of interest, USC is frequently characterised by the hyperactivation of the Her2/P13K/Akt/mTOR pathway (Cancer Genome Atlas Network, 2012, 2013; Zhao et al., 2013) and recent studies by our group showed that oncogenic mutations in the Pik3ca gene may not only confer resistance to Trastuzumab but also sensitize USC primary cell lines to the PIK3CA inhibitors such as Taselisib (English et al., 2013; Black et al., 2015; Lopez et al., 2015).

In this study, we first evaluated CCNE1 expression in USC by IHC using a tissue microarray (TMA) including 95 samples and then tested the in vitro and in vivo effectiveness of the CDK2/9 inhibitor CYC065 against multiple primary USC cell lines in vitro. Next, we evaluated the in vitro and in vivo activity of CYC065 and Taselisib as single agents or in combination against USCs harbouring amplification of CCNE1 and activating mutations in the Her2/P13K/Akt/mTOR pathway. We demonstrate for the first time that the dual targeting of CCNE1 and PIK3CA with CYC065 and Taselisib is synergistic against CCNE1-amplified/PIK3ca-mutated USC primary cell lines in vitro as well as in vivo. These results may have important implications for USC patients harbouring chemotherapy-resistant/recurrent disease.

**Tissue microarray cyclin E immunohistochemistry.** Study approval was obtained from the Ethical Institutional Review Board at Yale University according to the institutional guidelines. Tissue microarray was constructed using archived formalin-fixed, paraffin-embedded surgical pathology blocks from 95 previously diagnosed USC cases at the Department of Pathology, Yale University. Each tumour was represented in duplicates of 0.6 mm cores on the TMA. Cyclin E1 immunohistochemistry was performed on 4 μm TMA sections. Following deparaffinisation and rehydration, endogenous peroxidase was blocked in 3% H2O2. Steam and high pH (pH 9) were used for antigen retrieval. The slides were then incubated overnight at 4°C with cyclin E antibody (clone HE12, 1:400 dilution; Millipore, Billerica, MA, USA). EnVision system (Dako, Carpinteria, CA, USA) was used for secondary detection and the reactions were visualised with diaminobenzidine. Appropriate positive and negative controls were used. Only nuclear immunoreactivity was considered positive. Immunostaining was assessed using a semiquantitative scoring system, as follows: 0, negative (<5% of tumour cell nuclei staining); 1, weakly/focally (5–50% of tumour cell nuclei) positive; 2, strongly/diffusely (>50% of tumour cell nuclei) positive.

**Inhibitors.** Taselisib was purchased from Medchemexpress (Monmouth Junction, NJ, USA), whereas CYC065 was provided by Cyclacel. Both compounds were dissolved in DMSO as a 10 mM stock solution and diluted in culture medium immediately before use.

**Cell viability assay and synergism.** The effect of CYC065 on the viability and IC50 of USC-ARK-1, USC-ARK-2, USC-ARK-7, USC-ARK-4 and USC-ARK-6 USC primary cell lines was determined in flow-cytometry assays as described previously (Lopez et al., 2015). Briefly, tumour cells were plated in six-well plates and treated with a titration of CYC065 concentrations (i.e., ranging from 100 to 500 nM). After 72 h, cells were harvested, washed and stained with propidium iodide (PI; 5 μg ml−1) for flow cytometric counts. The percentage of viable cells was then normalised considering the vehicle-treated cells as 100% viable. Half-maximal inhibitory concentration values were determined using GraphPad Prism5 version 6 (GraphPad Prism software, San Diego, CA, USA). For drug combination studies, USC-ARK-1 and USC-ARK-2 cell lines were incubated with the combination of Taselisib and CYC065 at multiple paired concentrations including the IC50, the IC50/2 and the IC50/2 of each cell line to the corresponding drug (i.e., 10 nM of Taselisib and 198 nM of CYC065 for USC-ARK-1 and 50 nM of Taselisib and 62.5 nM of CYC065 for USC-ARK-2). Synergism was assessed by the combination index (CI), according to the mathematical method described by Chou and Talalay. CI values <1 define a synergistic activity of the combination treatment (Chou, 2006). The CI values were calculated using the CompuSyn software (CompuSyn, Paramus, NJ, USA).

**Cell-cycle analysis.** Uterine serous carcinoma cells were seeded in six-well tissue culture plates and 24 h later were treated with 100 nM of CYC065. After 48 h, treated and control cells were permeabilised with ice-cold 70% ethanol and fixed for 30 min at 4°C. After spining at 2000 r.p.m. for 5 min and discarding the supernatant, cells were resuspended in PBS1*. After additional spining at 2000 r.p.m. for 5 min, 100 μl ribonucleic acid (100 μg ml−1, Dnsase free; Sigma, St Louis, MO, USA) was added for 5 min incubation at room temperature, before exposure to PI (50 μg ml−1). CYC065-treated
and -untreated control cells were acquired with FACSCalibur, using Cell Quest software (BD Biosciences, San Jose, CA, USA) and were analyzed using the FlowJo software (Ashland, OR, USA).

**Knockdown of CCNE1.** USC-ARK-2 cell line was chosen as a representative CCNE1-amplified cell line to be tested in knockdown experiments. Cyclin-E1-specific siRNA oligonucleotides (i.e., CCNE1: 5'-GGAUGUUAGACUCCUUGAATT-3' (sense) and 5'-UUCAG GCAGUCACAUCCAG-3' (antisense)) and nonspecific MOCK siRNA duplexes were used as negative controls (purchased from Ambion, Waltham, MA, USA). Briefly, USC-ARK-2 cells were cultured in six-well plates and transfected with anti-CCNE1 siRNA duplexes at 10 nM in conjunction with 5 μl Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Mock transfections were used as negative controls. Uterine serous carcinoma cells were treated 24 h later with scalar doses of CYC065. After 72 h, cells were harvested and used either for RNA extraction or for cell viability assays.

**Immunoblotting.** Cells were seeded in Petri dishes and left to adhere overnight. The following day, cells were incubated with Taselisib (10 nM), CYC065 (200 nM) or the combination for 6 h. Cells adhere overnight. The following day, cells were incubated with Cells were seeded in Petri dishes and left to extraction or for cell viability assays.

**RESULTS**

**Cyclin-E1 transcript levels correlate with CNV analysis.** A recent NGS study by our group evaluated copy number variations (CNV) on 25 matched (tumour and normal tissue from the same patient) USC samples (Zhao et al, 2013). In agreement with the TCGA data in serous endometrial cancer, we found focal amplification of the CCNE1 gene on chromosome 19 in 48% of the USC cases (Supplementary Figure 1A). To assess whether the amplification of CCNE1 correlated with a higher expression of the CCNE1 transcript, the same whole-exome-sequenced USC samples described above were tested by real-time PCR. We found that USC samples with CCNE1 gain of function by CNV expressed significantly higher levels of CCNE1 transcript compared with USC samples found with copy neutral or loss of function of the gene (CCNE1-wt/loss USC; Supplementary Figure 1B; P = 0.0013).

**Tissue microarray immunohistochemistry and FISH results.** Next, we used IHC to evaluate the expression of CCNE1 at the protein level in 95 primary USC samples using a TMA. Cyclin E1 expression levels were scored from 0 to 2+, where 0 represented no staining, 1+ weak/focal staining and 2+ strong/diffuse staining. We found CCNE1 expression in 85 samples (89.5% of the total), with 33 (34.7%) of the USC demonstrating a score 1+ and 52 (54.8%) a score 2+. Of the CCNE1+ samples, 38 (44.7%) were from early-stage (stages I and II) patients, whereas 47 (53.3%) were from advanced-stage (stages III and IV) patients. None of the patients received neoadjuvant chemotherapy before tissue collection. Of the 10 samples in which CCNE1 staining was not detected (0 score) (10.5% of the total), 9 (90%) belonged to patients harbouring early-stage (stages I and II) disease. Representative IHC pictures of 0, a 1+ and a 2+ USC are depicted in Figure 1.

**Cyclin E1 overexpressing primary USC cell lines respond to the CDK2 inhibitor CYC065 in vitro.** To evaluate whether the overexpression of CCNE1 correlated with sensitivity to the CDK2/9 inhibitor CYC065, five USC primary cell lines were selected for in vitro proliferative and molecular assays based on their similar growth rate and differential expression of CCNE1 tested by real-time PCR, FISH and western blot (Table 1 and Supplementary Figures 2 and 3). We consistently found USC cell lines expressing high CCNE1 mRNA and protein levels to be significantly more sensitive to treatment with CYC065 in vitro when compared with low CCNE1-expressing cell lines (Figure 2A and B; IC50: mean ± s.d. = 124.1 ± 57.8 nM in CCNE1-overexpressing USC cell lines vs 415 ± 117.5 nM in CCNE1 low expressors, respectively; P = 0.0003). Importantly, low concentrations of CYC065 (i.e., 100 nM) caused an arrest in the G1 phase of the cell cycle only in the CCNE1-
overexpressing USC cell lines (i.e., USC-ARK-2, USC-ARK-7) (Figure 2C).

In recent studies, the 6–8 h pulse treatment with CYC065 at 500–1000 nM has been reported to rapidly induce cell death via CDR9 inhibition in acute myeloid leukaemia (AML) and breast cancer cell lines (MacKay et al, 2015; Saladino et al, 2015). Consistently, we observed about two-fold increase in the expression of CCNE1 (fold increase annexin V/PI-positive cells = 1.78 ± 0.3 and 2.25 ± 0.7) following 6 h treatment of the USC-ARK-7 primary cancer cell line with the same concentrations of CYC065 in vitro (fold increase annexin V/PI-positive cells = 1.78 ± 0.3 and 2.25 ± 0.7 after treatment of cells with 500 and 1000 nM of CYC065, respectively; Supplementary Figure 4).

Knockdown of CCNE1 confers resistance to CYC065 in vitro. Next, to determine the specificity of CYC065 in targeting CCNE1 in the overexpressing USC cell lines, the expression of CCNE1 in USC-ARK-2 was knocked down using siRNA technology. Cyclin E1 knockdown resulted in a 9.29-fold increase in CYC065 IC50 compared with the control (cells transfected with MOCK) following treatment with scalar concentrations of the drug (Figure 2D, P = 0.021). Interestingly, we found that CCNE1 knockdown caused a significant inhibition of tumour cells' growth compared with MOCK-transfected cells even without treatment (Supplementary Figure 5; % of growth inhibition: mean ± s.d. = 49.5 ± 14.4, P = 0.027). These data suggest a high dependency of USC to CCNE1 for cell proliferation.

CYC065 inhibits tumour growth in xenografts established from CCNE1-amplified USC. To evaluate the therapeutic potential of CYC065 as a single agent, USC-ARK-2-derived xenografts were treated daily with CYC065 (22.5 mg kg\(^{-1}\)) for a 3-week period. Tumour size and mouse weight were recorded two times a week. The daily administration of CYC065 resulted in a significant reduction of tumour growth compared with the vehicle-treated mice (Figure 2E, P = 0.012 starting at day 9 of the treatment). No significant weight loss was reported during the entire treatment period (Figure 2F).

Synergistic effect of the combination of CYC065 and Taselisib in vitro. Because a recent NGS analysis from our group (Zhao et al, 2013) demonstrated that up to 91.7% of CCNE1-amplified USC may also carry mutations in genes involved in the Her2/PI3K/AKT/mTOR pathway (Supplementary Table 1), we next tested the activity of CYC065 and Taselisib alone or in combination using two USC primary tumour models (i.e., USC-ARK-1, a CCNE1/c-erbB2/pik3ca-amplified and pik3ca-mutated cell line and USC-ARK-2, a CCNE1/c-erbB2/pik3ca-amplified cell line harbouring wild-type Pik3CA gene; Supplementary Table 1). As shown in Figure 3A, the incubation of USC-ARK-1 and USC-ARK-2 cells with the combination of CYC065 and Taselisib was significantly more effective in inhibiting tumour growth than each of the single agents in both cell lines (Figure 3A, % viable cells: mean ± s.d. = 65.8 ± 7.9, 75.4 ± 11.3 and 48 ± 7.6 for the CYC065, Taselisib and the combination, respectively, for USC-ARK-1; P<0.05), and 48.9 ± 4.6, 36.7 ± 5.5 and 15.9 ± 4.05 for CYC065, Taselisib and the combination, respectively, for USC-ARK-2; P<0.05). Importantly, when we evaluated the effect of the combination of CYC065 and Taselisib at multiple paired concentrations using the CompuSyn software, we found a synergistic activity of the combination of CYC065 and Taselisib in both USC models (CI values: 0.93, 0.81, 0.71, 0.65 for Fa = 0.5, 0.75, 0.9 and 0.95, respectively, in USC-ARK-1 and 0.45, 0.46, 0.54 and 0.64 for Fa = 0.5, 0.75, 0.9 and 0.95, respectively, in USC-ARK-2).

Immunoblot analysis after combination treatment with CYC065 and Taselisib. Western blot analysis was carried out to investigate the molecular basis of the drug synergism after treatment of USC-ARK-1 and USC-ARK-2 with CYC065 and Taselisib. Changes in the total Rb, HER2/neu and S6 and in their phosphorylation status were evaluated after 6 h of treatment with 200 nM of CYC065,

Table 1. Primary USC cell lines selection

| USC cell line | Histology | Doubling time (h) | CCNE1 CNV | RT-PCR (ΔCT) |
|--------------|-----------|------------------|-----------|--------------|
| USC-ARK-1    | USC       | 23.4             | Gain      | 5.415        |
| USC-ARK-2    | USC       | 18.2             | Gain      | 4.991        |
| USC-ARK-7    | USC       | 30.6             | Gain      | 4.141        |
| USC-ARK-4    | USC       | 17.1             | N/A*      | 8.036        |
| USC-ARK-6    | USC       | 28.5             | Copy neutral | 7.335 |

Abbreviations: CCNE1 = cyclin E1; CNV = copy number variation; FISH = fluorescent in situ hybridisation; RT-PCR = reverse transcription–PCR; USC = uterine serous carcinoma.

*Normal matched sample was not available for USC-ARK-4. Cyclin-E1 copy number was evaluated by FISH. The sample was found copy neutral (Supplementary Figure 2).

Figure 1. Cyclin E1 expression by IHC in USC. Representative immunohistochemical staining for CCNE1 on USC samples scoring 0 (A), 1+ (B) and 2+ (C). A representative negative control (i.e., TMA USC sample stained without the primary antibody, D) and a representative positive control (i.e., cell block from a cell line (ARK2) with known CCNE1 amplification by fluorescent in situ hybridisation (FISH), E) are also shown. (All images at ×200 original magnification.)
10 nM of Taselisib or the combination. Changes in the expression of CCNE1 were also examined. As shown in Figure 3B, incubation with Taselisib was able to induce a decrease in the phosphorylation of S6 in both cell lines, whereas CYC065 was effective in inhibiting Rb phosphorylation in USC-ARK-1 cell line but not in USC-ARK-2. Importantly, the combination of CYC065 and Taselisib was highly effective in inhibiting pRb and pS6 in both cell lines (Figure 3B). No changes in total HER2/neu, pHER2/neu and total S6 were...
observed. Conversely, decreased total Rb was reported in USC-ARK-1 but not in USC-ARK-2 following treatment with the combination of CYC065 and Taselisib. An increased expression of CCNE1 was also observed after incubation with CYC065 and with the combination of CYC065 and Taselisib in both USC cell lines.

**In vivo activity of CYC065 and Taselisib on USC xenografts.** To assess whether the synergism observed in our in vitro assays also resulted in an improved therapeutic effect in vivo, Scid mice bearing USC-ARK-1-derived xenografts were treated with CYC065 (q.d. for 3 weeks), Taselisib (5 days a week for 3 weeks) or with the combination of CYC065 and Taselisib. An increased expression of CCNE1 was also observed after incubation with CYC065 and with the combination of CYC065 and Taselisib in both USC cell lines.

The weight of the animals in the different groups was monitored two times a week during the course of the experiment. No significant differences were reported between the different groups, although a slight decrease in mice weight was observed in the combination group starting from day 19 of the treatment (Figure 4B; \( P < 0.05 \)).

**DISCUSSION**

Uterine serous carcinoma is the most aggressive variant of endometrial cancer (Siegel et al, 2015). Owing to the intrinsic resistance of this tumour to chemotherapy, patients diagnosed with advanced-stage USC have an extremely poor prognosis (Schwab and Santin, 2015). The development of novel and more effective therapeutic strategies to target chemotherapy resistant/recurrent USC therefore remains of paramount importance.

Multiple comprehensive NGS studies performed by us as well as other groups have recently analysed the genetic landscape of USC (Kuhn et al, 2012, 2014; Cancer Genome Atlas Research Network et al, 2013; Zhao et al, 2013). The results of these studies
groups, although a slight decrease in mice weight was observed in the Taselisib and in the combination groups, respectively (P = 0.05).

Cyclin E1, an activator of CDK2, is known to have a critical role in the regulation of the G1–S phase transition in mammalian cells. Once formed, the CCNE1/CDK2 complex promotes the phosphorylation of several proteins involved in cell cycle progression (i.e., p27 and Rb), DNA replication (i.e., replication factors A and C) and histone biosynthesis (i.e., NPAT) (Reed, 1996; Ekholm and Reed, 2000; Ma et al., 2000; Okuda et al., 2000; Sever-Chronoeeos et al., 2001; Hubalek et al., 2004; Whittaker et al., 2004; Shapiro, 2006; Bose et al., 2013). Overexpression of CCNE1 has been associated with a broad spectrum of human malignancies and found to correlate with worse prognosis (Sui et al., 2001; Rosen et al., 2006; Scaltriti et al., 2011; Lundgren et al., 2015). On the basis of these data, the use of CDK inhibitors (CDKi) to block the activity of the CCNE1/CDK2 complex as a therapeutic strategy for the treatment of multiple cancers has been extensively investigated over the past two decades (Asghar et al., 2015). First-generation CDKi (including alvocidib and seliciclib) tested in phase I and II clinical trials have shown limited potency or selectivity for CDK2 (Heer et al., 2008; Le Tourneau et al., 2010; Nemunaitis et al., 2013). Second-generation CDKi with varying CDKi profiles including increased potency against CDK2 are currently undergoing preclinical and/or clinical evaluation (Senderowitz et al., 1998; Taylor-Harding et al., 2015; Yang et al., 2015).

Cycloabatin 065 is a recently developed second-generation CDK9 inhibitor with reported activity against AML and CCNE1-amplified breast cancer (Scaltriti et al., 2011; MacKay et al., 2015; Saladino et al., 2015). Accordingly, in the current study, we initially evaluated the expression of CCNE1 in USC by IHC in a large number of USC using a TMA. We found the majority of USC (89.5%) to express CCNE1 at moderate or high levels, strongly supporting a correlation between gene amplification, mRNA and protein expression results, and, importantly, suggesting that a large number of USC may be addicted to overactivation of the CCNE1 pathway. Interestingly, CCNE1 expression was found to be more prevalent in late stages (stages III and IV) USC, suggesting a preferential expression of this protein during the progression of the disease. On the basis of these results, we initially investigated the therapeutic potential of Cycloabatin 065 against CCNE1-amplified USC cell lines as single agent. We found elevated CCNE1 expression to significantly correlate with sensitivity to Cycloabatin 065 in vitro and that the treatment of USC cell lines with Cycloabatin 065 caused accumulation of cells in the G1 phase of the cell cycle specifically in CCNE1-amplified USC cell lines. Knockdown of CCNE1 resulted in strong cell growth inhibition and in resistance to Cycloabatin 065 supporting a relationship between high CCNE1 expression in USC and sensitivity to Cycloabatin 065. These data are therefore in agreement with the results of other groups demonstrating an ‘oncogene addition’ of multiple human tumours including ovarian cancer (i.e., a tumour histologically similar to USC) to CCNE1 expression (Etemadmoghadam et al., 2010, 2013). Treatment of USC cell lines with higher concentrations of Cycloabatin 065 also induced apoptosis as has been reported for Cycloabatin 065 in other cancer cell lines (MacKay et al., 2015; Saladino et al., 2015). Importantly, Cycloabatin 065 was effective in vitro in decreasing tumour growth in xenografts derived from CCNE1-amplified USC. Taken together, these data suggest a high dependency of USC cell proliferation on the CCNE1 pathway and promising activity of Cycloabatin 065 in targeting the CCNE1-amplified USC cell lines both in vitro as well as in vivo.

Studies by our research group have recently shown that USC may be extremely sensitive in vitro to compounds that selectively target the Her2/Pi3K/Akt/mTor pathway (English et al., 2013; Lopez et al., 2014, 2015; Schwab et al., 2014). In these studies, however, treatment with highly targeted Pi3K/Akt agents was found to be only transiently effective in vivo in controlling the growth of USC xenograft models harbouring HER2/neu gene amplification or oncogenic pi3kca mutations. These in vivo data in animals therefore mirror long-term data in human patients where, after initial responses, progression is generally detected few months after starting targeted treatment in a large number of cancer patients (Engelman and Janne, 2008). Importantly, recent preclinical data strongly suggest that dual-targeting of HER2/Pi3KCA with neratinib (a pan-c-erb inhibitor) and Taselisib may be highly synergistic and able to achieve durable regression of established USC xenografts in vivo (Lopez et al., 2015).

On the basis of these data and the recent observation that over 90% of CCNE1-amplified USC may also harbour oncogenic alterations in the genes encoding for proteins of the Her2/Pi3K/akt/mTor pathway (i.e., pi3kca, c-erbB2, akt1), we next evaluated the activity of the combination of Cycloabatin 065 and Taselisib against primary USC cell
the combination of Taselisib and CYC065 was highly effective in signalling assessed by immunoblotting experiments showed that the combination of Taselisib and CYC065 was highly effective in inhibiting the phosphorylation of S6 and Rb, the downstream effectors of PI3K and CCNE1/CDK2, respectively, and, importantly, significantly more efficacious than each of the single agents when used alone in inhibiting tumour growth in xenograft models derived from a CCNE1-amplified, pik3ca-mutated USC cell line.

As CDKi target proteins that are essential for the proliferation and the survival of normal as well as tumour cells, their use at high dose as single agents may be associated with significant toxicity (Senderowicz et al, 1998; Shapiro, 2006; Heath et al, 2008). A more rationale use of CDKi in USC patients might therefore be a lower dose in combination with other molecularly targeted agents acting in synergy. Consistent with this view, Palbociclib, an inhibitor of CDK4 and 6, has recently been shown to be clinically effective (and for this reason approved by the FDA) when used in combination with letrozole for the treatment of postmenopausal patients with estrogen receptor-positive, Her2-negative advanced breast cancer (Beaver et al, 2015). Taken together, these data combined with our results strongly support the view that CYC065 may represent a promising novel compound to be considered either alone or in combination with selective Her2/PI3K/Akt/mTor inhibitors for the treatment of USC patients harbouring chemo-therapy-resistant/recurrent CCNE1-amplified endometrial cancer.

ACKNOWLEDGEMENTS

This work was supported, in part, by R01 CA154460-01 and U01 CA176067-01A1 grants from NIH, the Deborah Bunn Alley Foundation, the Tina Brozman Foundation, the Discovery to Cure Foundation and the Guido Berlucchi Foundation (to ADS). This investigation was also supported by NIH Research Grant CA-16359 from the NCI and by the Italian Ministry of Health Grant RF-2010-2313497 (to ADS).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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