Supplementary material and methods

Drugs

Palbociclib were purchased from Selleckchem for *in vitro* and LC Laboratories for *in vivo* analyses, respectively. MLN0128 were purchased from LC Laboratories. For *in vitro* experiments, they were dissolved in dimethyl sulfoxide (DMSO). For *in vivo* studies, a palbociclib stock solution was prepared with phosphate-buffered saline (PBS). MLN0128 was dissolved using 5% N-methyl-2-pyrrolidone, 15% polyvinyl pyrrolidone in water (MLN0128 vehicle).

Cell proliferation assay and combination index analysis

To quantify the effects of drugs on cell proliferation, we initially performed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). MB231, MB453, MB468, and CAL148 were seeded into 96-well plates at densities of 2000 – 5000 cells per well in 100 μl of DMEM. After 24 h incubation at 37 ˚C, cells were treated with palbociclib, and/or MLN0128. DMSO was used as a vehicle control. After replacing media with the drugs, cells were cultured up to 120 h. After 24, 72 and 120 h, MTT reagent was added to each well at a concentration of 1 mg/ml in phenol red free DMEM and incubated for 1 h at 37 ˚C. After incubation, the reagent was removed, and then 100 μl of DMSO was added to each well. The absorbance at 570 nm was measured using a 96-well plate reader. A combination index analysis was performed using CalcuSyn software, version 2 (Biosoft) and the Chou-Talalay method (Cancer Res, 2010).

Colony formation assay

This assay was performed to confirm the growth suppression effects of the drugs. Cells were seeded in 6-well plates at a level of 1000 cells for MB231 or 2000 cells for MB453 per well in 2 ml DMEM. After attachment of cells to the plates, the drug-free media was changed to media containing one of the following: control (vehicle), palbociclib, MLN0128, or palbociclib + MLN0128. The culture media were replaced every 3 days with DMEM containing fresh drugs. Cells were incubated for 7 days in MB231 or for 21 days in MB453. Cells were then washed twice with PBS and incubated with 0.5% crystal violet (Sigma-Aldrich) in methanol at room temperature (RT) for 30 minutes. Colony numbers were counted manually.

Cell cycle analysis

The growth suppression effects of the two drugs were also evaluated through cell cycle analysis. Single cells were collected from cell culture and fixed with 70% ethanol at -20 ˚C. After fixation, single-cell suspensions were incubated with 20 μg/ml of RNaseA (Thermo Fisher Scientific) in PBS at RT for 30 minutes and then stained with 100 μg/ml of propidium iodide (Life Technology) at 4 ˚C overnight. Flow cytometric analysis was conducted using a CyAn ADP cytometer (Dako) and analyzed with FlowJo software (TreeStar). These analyses were performed by the Analytical Cytometry Core facility at City of Hope

Western blot analysis

Cells were seeded into 60 mm petri dishes and incubated at 37 ˚C for 24 h. After incubation, cells were treated with palbociclib and/or MLN0128 for 48 h. Cells were lysed using lysis buffer [0.15 M NaCl, 0.5% sodium deoxycholate, 0.1% Nonident P40, 50 mM Tris-HCl pH 7.5, cOmplete™ protease inhibitor cocktail (Roche), and Halt™ phosphatase inhibitor cocktail (Thermo Fisher Scientific)], then mixed with loading sample buffer (50 mM Tris-HCl pH 8.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 10% 2-mercaptoethanol), and heated at 100 ˚C for 5 minutes. The lysates were loaded into polyacrylamide gels, subjected to electrophoresis at 150 V for 60 minutes, and transferred to a polyvinylidene difluoride membrane by wet transfer at 300 mA for 60 minutes. The membranes were blocked in blocking buffer (5% non-fat milk in TBST) at RT for 60 minutes and incubated with appropriate primary antibody in 5% non-fat milk or bovine serum albumin in TBST at 4 ˚C overnight. Then the membranes were incubated with the appropriate secondary antibody in blocking buffer at RT for 60 minutes. The signal was detected with Clarity™ Western ECL Blotting Substrate (Bio-Rad) using a ChemiDoc™MP imaging system (Bio-Rad).

Quantitative PCR analysis

Each cDNA was synthesized from 1 μg of RNA. Real-time reverse transcription-PCR was carried out using CFX Connect™ Real-Time System (Bio-Rad). cDNA samples prepared from MB231 and MB453 were mixed in 20 μl reactions containing SYBR Green FastMix (Quantabio) and each primer: ACTB 5′-GCCCTGGACTTCGAGCAAGA-3′ (forward) and 5′-ACGGAGTACTTGCGCTCAGG-3′ (reverse), and CCNE1 5′-ACACCATGAAGGAGGACGGC-3′ (forward) and 5′-TCAGGTGTGGGGATCAGGGA-3′

(reverse). The expression levels of CCNE1 were determined relative to the expression level of ACTB.

Antibodies

For Western blotting, anti-pRb (#9309), anti-phospho-pRb (Ser807/811) (#9308), anti-mTOR (#2972), anti-phospho-mTOR (Ser2448) (#2971), anti-p70 S6 kinase (#9202), anti-phopho-p70 S6 kinase (Thr389) (#9234), anti-phopho-S6 (Ser235/236) (#4858), anti-cyclin E1 (#4129), and anti-cyclin E2 (#4132) antibodies were purchased from Cell Signaling Technology, and anti-cyclin D1 (sc-20044) and anti-β-actin (sc-47778) antibodies were purchased from Santa Cruz Biotechnology. All antibodies were used at 1:1000 dilutions. For IHC, anti-Ki67 (M7240) was purchased from Dako.

Statistical analysis

Unless otherwise described, *in vitro* data are presented as mean ± SD. *In vivo* animal data are presented as mean ± SEM. Significant differences ($p < 0.05$) between the 4 groups were determined by one-way ANOVA with Tukey's multiple comparison post-hoc test.