# Combined inhibition of ACLY and CDK4/6 reduces cancer cell growth and invasion

BRIAN CHRISTOPHER VELEZ*, CHRISTOPHER PAUL PETRELLA*, KATHLEEN HELEN DiSALVO, KEYI CHENG, REBECCA KRAVTSOV, DORINA KRASNIQI and NANCY ANN KRUCHER

Department of Biology, Pace University, Pleasantville, NY 10570, USA

Received August 8, 2022; Accepted November 23, 2022

DOI: 10.3892/or.2022.8469

**Abstract.** The use of small molecule kinase inhibitors, which target specific enzymes that are overactive in cancer cells, has revolutionized cancer patient treatment. To treat some types of breast cancer, CDK4/6 inhibitors, such as palbociclib, have been developed that target the phosphorylation of the retinoblastoma tumor suppressor gene. Acquired resistance to CDK4/6 inhibitors may be due to activation of the AKT pro-survival signaling pathway that stimulates several processes, such as growth, metastasis and changes in metabolism that support rapid cell proliferation. The aim of the present study was to investigate whether targeting ATP citrate lyase (ACLY), a downstream target of AKT, may combine with CDK4/6 inhibition to inhibit tumorigenesis. The present study determined that ACLY is activated in breast and pancreatic cancer cells in response to palbociclib treatment and AKT mediates this effect. Inhibition of ACLY using bempedoic acid used in combination with palbociclib reduced cell viability in a panel of breast and pancreatic cancer cell lines. This effect was also observed using breast cancer cells grown in 3D cell culture. Mechanistically, palbociclib inhibited cell proliferation, whereas bempedoic acid stimulated apoptosis. Finally, using Transwell invasion assays and immunoblotting, the present study demonstrated that ACLY inhibition blocked cell invasion, when used alone or in combination with palbociclib. These data may yield useful information that could guide the development of future therapies aimed at the reduction of acquired resistance observed clinically.

*Correspondence to:* Dr Nancy Ann Krucher, Department of Biology, Pace University, 219 Dyson Hall, 861 Bedford Road, Pleasantville, NY 10570, USA
E-mail: nkrucher@pace.edu

*Contributed equally

*Key words:* CDK4/6, resistance, palbociclib, ATP citrate lyase, bempedoic acid

## Introduction

Dysregulation of the tumor suppressor protein retinoblastoma (Rb) is commonly found in human cancer. Hyperphosphorylation rather than mutation of Rb is thought to promote tumorigenesis (1). The discovery over 20 years ago that phosphorylation of Rb by CDKs promotes cellular proliferation led to the development of CDK inhibitors; however, the early compounds failed due to lack of specificity or had low affinity for CDK4/6 enzymes (2). Subsequently, three selective inhibitors of CDK4/6 have been successful in patients. Palbociclib (Palb), ribociclib and abemaciclib have been approved by the Food and Drug Administration (FDA). These treatments inhibit CDK4/6 to reduce Rb phosphorylation, exerting anti-proliferative activity in breast cancer (3). Furthermore, studies using preclinical models demonstrate that the efficacy of these treatments is dependent on Rb (4). These CDK4/6 inhibitors have been shown to benefit patients with estrogen receptor-positive, HER2-negative advanced breast cancer (3). Unfortunately, most patients acquire resistance to treatment with CDK4/6 inhibitors and research into the mechanisms that trigger resistance is ongoing (5). It is thought that acquired resistance is due to the activation of alternative growth promoting pathways in response to the treatment. The PI3K/AKT/mTOR pathway has been implicated in acquired resistance to targeted therapies in breast cancer (6), and several mechanistic studies in breast and pancreatic cancer have described the activation of this pathway during treatment resistance (7-13). Not only does the PI3K/AKT/mTOR pathway stimulate cell growth, survival and proliferation, but it also enhances tumor aggressiveness by promoting epithelial-mesenchymal transition (EMT) (14,15). One downstream effector of this pathway is the enzyme ATP citrate lyase (ACLY). ACLY is activated by AKT phosphorylation at serine 455 (16). This enzyme connects glucose and lipid metabolism as it converts excess cytosolic citrate in cancer cells to acetyl-CoA, which is the precursor to fatty acid and cholesterol synthesis (17). In keeping with the requirement of cancer cells for abundant fatty acids needed for membrane biosynthesis, cancer cells exhibit elevated ACLY activity, and ACLY gene knockdown suppresses tumor cell growth (18,19). Recently, lipid reprogramming has been proposed to be essential in the mechanism of resistance to kinase inhibitors in breast cancer (20). In 2019, an inhibitor of ACLY, bempedoic acid (BA), was FDA-approved to reduce levels of low-density

lipoprotein cholesterol in patients (21). Thus, to target ACLY activity in cancer cells, BA was utilized to investigate the role of ACLY in tumorigenesis. The present study aimed to determine the combined effect of ACLY inhibition using BA and CDK4/6 inhibition using Palb on the proliferation and EMT/invasion of cancer cells.

## Materials and methods

*Cell culture*. Cell culture materials were obtained from Gibco (Thermo Fisher Scientific, Inc.), unless otherwise indicated. The MDA-MB-231 (cat. no. HTB-26), T47D (cat. no. HTB-133), MCF7 (cat. no. HTB-22), Panc1 (cat. no. CRL-1569), MIAPaCa-2 (cat. no. CRL-1420) and HT-1080 (cat. no. CCL-121) cell lines were obtained from American Type Culture Collection. All cell lines were handled using good cell culture practices and utilized within 4 months of receipt. MCF7 cells were grown in Eagle's Minimum Essential Medium supplemented with 10% FBS and 1X Penicillin-Streptomycin-Glutamine (PSG), containing 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 2 mM glutamine. All other cells used in the present study were grown in DMEM, supplemented with 10% FBS and 1X PSG. Cells were routinely maintained at 37˚C in a humidified, 5% $CO_2$-containing atmosphere and were split two to three times weekly to maintain sub-confluent cultures. Cultures were routinely tested for mycoplasma contamination using the MycoFluor™ Mycoplasma Detection Kit (Invitrogen; Thermo Fisher Scientific, Inc.). 3D cell culture was performed either by placement of cells in plates pre-coated with Matrigel (Corning, Inc.) at room temperature (RT) for 2 h before cell addition, as previously described (22,23) or by growing cells in ultra-low attachment plates according to the manufacturer (Corning, Inc.). A total of 7 days after plating, cell spheres formed and treatments (0.1% DMSO, 1 $\mu$M Palb, 25 $\mu$M BA or the combination of Palb + BA were administered at 37˚C for up to 96 h.

*Western blotting*. Cell extracts were prepared using MDA-MBA-231, Panc1 or HT1080 cells after washing cells in ice-cold TBS [25 mM Tris-HCl (pH 8.0) and 150 mM NaCl], followed by cell lysis for 15 min at 4˚C in EBC buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl and 0.5% Nonidet P-40] containing 10 $\mu$g/ml protease inhibitors (aprotinin and PMSF). The lysates were cleared by centrifugation at 13,000 x g for 10 min at 4˚C. Protein concentration was determined using the Bradford protein assay. Electrophoresis was performed using 4-20% gradient SDS-polyacrylamide gels containing 30 $\mu$g total cell protein in each sample lane. Following electrophoresis, the proteins were transferred to nitrocellulose membranes. Residual protein binding sites on the nitrocellulose membranes were blocked by incubation with TBS-0.5% Tween-20 (TBST) containing 4% non-fat dry milk for 30-60 min at RT. Subsequently, the nitrocellulose membranes were incubated in TBST containing 2% non-fat dry milk and 1 $\mu$g/ml primary antibody overnight at 4˚C. After three washes with TBST (5 min/wash), the nitrocellulose membranes were probed with HRP-conjugated anti-IgG antibodies [1:2,000; cat. nos. 1031-05 (anti-mouse) and 4050-05 (anti-rabbit); SouthernBiotech] for 1.5 h at RT and developed using enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The following primary antibodies were used in the present study (all 1:1,000 dilution): AKT (cat. no 4691), phosphorylated (p)-AKT (ser473) (cat. no. 4060), ACLY (cat. no 13390), p-ACLY (ser455) (cat. no. 4331), p-Rb (ser780) (cat. no. 8180), cleaved poly (ADP-ribose) polymerase (cPARP; cat. no. 9541), p-c-Jun (cat. no. 2361), c-Jun (cat. no. 15683), proliferating cell nuclear antigen (PCNA; cat. no. 13110), Snail (cat. no. 3879), Slug (cat. no. 9585), zinc finger E-box-binding homeobox 1 (ZEB1; cat. no. 3396), E-cadherin (cat. no. 3195), N-cadherin (cat. no. 13116) and Vimentin (cat. no. 5741) (all from Cell Signaling Technology, Inc). In addition, β-actin (cat. no. A1978; Sigma-Aldrich; Merck KGaA), cyclin D3 (cat. no. 610279; BD Biosciences), Rb (cat. no. sc-102) and mini-chromosome maintenance complex component 7 (Mcm7; cat. no. sc-9966) (both from Santa Cruz Biotechnology, Inc.) were used. Immunoblotting results were quantified using ImageJ software v0.5.6 (National Institutes of Health).

*Transfection*. MDA-MB-231 or Panc1 cells were transfected with AKT small interfering RNA (cat. no. 6211; Cell Signaling Technology, Inc.) or NT RNA as a control (cat. no. 6568; Cell Signaling Technology, Inc.) at 100 nM at 37˚C for 48 h prior to cell lysis, using Lipofectamine RNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Transfections were performed according to the manufacturer's instructions.

*Viability, proliferation and apoptosis assays*. Palb (PD-0332991; cat. no. S1116) and BA (ETC-1002; cat. no. S7953) were obtained from Selleck Chemicals, and were used within 3 months of receipt. BA was dissolved in DMSO, while Palb was dissolved in water. Dose-response curves were generated to identify the concentration of each drug required to reduce cell numbers between 20-30% after a 96-h treatment at 37˚C compared with cells treated with water or DMSO (data not shown). The suitable concentrations determined were 1 $\mu$M Palb and 25 $\mu$M BA. MCF7 or MDA-MB-231 cells (6,000/well), T47D cells (12,000/well) and Panc1 or MIAPaCa-2 cells (3,000/well) were plated in 96-well plates and allowed to proliferate for 24 h. Cells were treated at 37˚C for 96 h with full culture medium as control, DMSO (0.1%) or Palb or BA alone or in combination with n=6. To measure viability, the following assays were employed (all from Promega Corporation; all assays performed with n=6 or n=8): CellTiter-Fluor Cell Viability Assay (cat. no. G6080), utilized on T47D, MCF7, MDA-MBA-231, Panc1 and MiaPaCa2 cells; RealTime-Glo MT Cell Viability assay (cat. no. G9711), utilized on MDA-MBA-231 and Panc1 cells; or the CellTiter-Glo 3D Cell Viability Assay (cat. no. G9681), utilized on MDA-MBA-231 cells. To determine toxicity and apoptosis, additional Promega assays were employed: the CellTox Green Cytotoxicity Assay (cat. no. G8741), utilized on MDA-MBA-231 cells; and the RealTime-Glo Annexin V Apoptosis and Necrosis Assay (cat. no. JA1000), utilized on T47D and MDA-MBA-231 cells. The Annexin V assay was utilized to measure apoptosis 48 h after addition of the aforementioned agents and presented as Annexin V (absorbance)/cell number (fluorescence). The cell number was determined using the CellTiter-Fluor Cell Viability Assay. Acetyl-CoA (Sigma-Aldrich; Merck KGaA) was used to show target validation of BA in MDA-MBA-231 cells in CellTox Green Cytotoxicity assays at a concentration

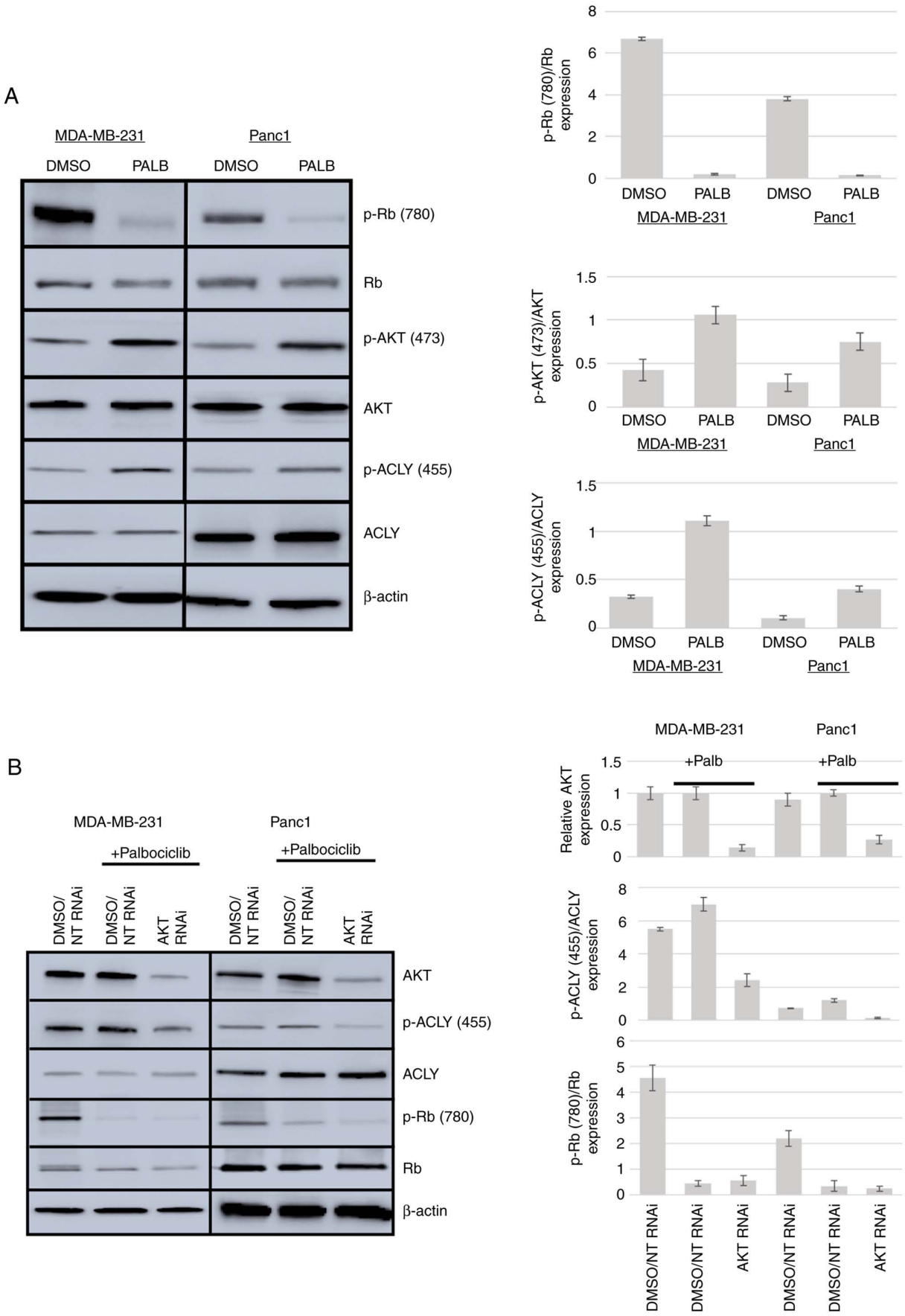


Figure 1. Palb activation of ACLY is dependent on AKT. (A) MDA-MB-231 and Panc1 cells were treated with 0.1% DMSO or 1 $\mu$M P for 96 h. Cellular protein was quantified using Bradford assays and equal amounts of protein were separated by SDS-PAGE. (B) MDA-MB-231 or Panc1 cells were treated with 0.1% DMSO and NT RNA as a control. Cells treated with 1 $\mu$M Palb for 96 h were also subjected to AKT knockdown. Protein analysis, western blotting and antibodies utilized was as described in the Materials and methods. Results shown were repeated twice, and Palb, palbociclib; NT, non-targeting; ACLY, ATP citrate lyase; Rb, retinoblastoma; RNAi, RNA interference; p, phosphorylated.

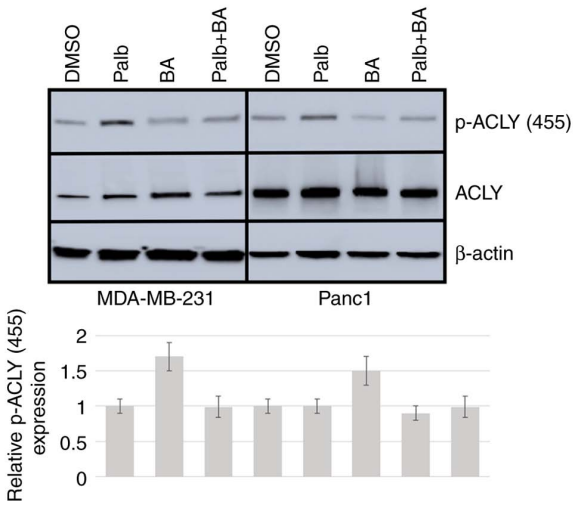


Figure 2. BA reverses Palb-induced activation of ACLY. MDA-MB-231 and Panc1 cells were treated with 0.1% DMSO as a control, 1 $\mu$M Palb, 25 $\mu$M BA or the combination of Palb + BA for 96 h followed by western blotting. Results shown were repeated twice. BA, bempedoic acid; Palb, palbociclib; ACLY, ATP citrate lyase; p, phosphorylated.

of 1 mM and was administered at the time of BA addition at 37˚C. Proliferation was monitored using the 5-bromo-2-deoxyuridine (BrdU) Cell Proliferation Assay Kit (cat. no. 6813; Cell Signaling Technology, Inc.). In the BrdU assay, cells were treated with the aforementioned agents for 72 h, and cells were fixed using 4% formaldehyde in PBS for 30 min at RT and a mouse monoclonal antibody to BrdU (dilution, 1:1,000) was applied, followed by the use of an anti-mouse HRP-linked antibody (dilution, 1:1,000) that recognizes the bound detection antibody. The HRP substrate 3,3',5,5'-tetramethylbenzidine was added to develop color. The magnitude of the absorbance (450 nm) corresponding to the developed color is proportional to the quantity of BrdU incorporated into cells, which is a direct indication of cell proliferation. All assays were performed as directed by the manufacturer.

*Invasion assays*. HT1080, Panc1 or MDA-MB-231 cells were treated with the 0.1% DMSO, 25 $\mu$M BA, 1 $\mu$M Palb or the combination of BA + Palb for 24 h, washed in serum-free medium, counted, and 50,000 cells were plated in 24-well plate Transwell chamber inserts (pore size, 8.0 $\mu$m) that were pre-coated with Matrigel at RT for 2 h before cell addition, while the lower compartment contained medium in the absence or presence of FBS as a chemoattractant. After incubation for 24 h at 37˚C, cells on the upper surface of the filter were removed and cells that were motile or invasive on the lower surface of the filter were quantified by staining with 10 $\mu$M Calcein AM (cat. no. C3100MP; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C followed by measurement of fluorescence using a microplate reader (GloMax; Promega Corporation).

*Statistical analysis*. All experiments performed in the present study were conducted two to three times. Numerical data are presented as the mean ± standard deviation. All data from each experiment were analyzed with either unpaired Student's t-test for the comparison between two groups or with one-way analysis of variance and Tukey's post hoc test for multiple comparisons using SPSS 19.0 software (IBM Corp.) as indicated. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Palb activates ACLY via AKT*. To determine whether ACLY, a substrate of AKT, was activated in response to Palb treatment, MDA-MB-231 cells as a model of breast cancer and Panc1 cells as a model of pancreatic cancer were used. These cell types were treated with 1 $\mu$M Palb and CDK4/6 inhibition was verified by measuring the phosphorylation of Rb. Phosphorylation of AKT at Ser473 and ACLY at Ser455 was evaluated by western blotting to reveal activation of these enzymes. Fig. 1A shows that both AKT and ACLY were activated in MDA-MB-231 and Panc1 cells in response to CDK4/6 inhibition. In MDA-MB-231 cells, Palb caused a 92% reduction in Rb phosphorylation at amino acid (aa) 780, a 2.6-fold increase in AKT phosphorylation at aa 473 and a 3.6-fold increase in ACLY phosphorylation at aa 455 relative to pan levels of AKT and ACLY. In Panc1 cells, Palb caused a 90% reduction in Rb phosphorylation, a 3-fold increase in AKT phosphorylation at aa 473 and a 4-fold increase in ACLY phosphorylation at aa 455 relative to the levels of AKT and ACLY, respectively. Furthermore, the activation of ACLY stimulated by Palb was dependent on AKT expression in both cell types. In MDA-MB-231 cells, knockdown of AKT resulted in an 86% reduction of AKT expression and a 55% decrease in ACLY activation compared with the control. In Panc1 cells, knockdown of AKT resulted in a 73% reduction of AKT expression and a 68% decrease in ACLY activation compared with the control (Fig. 1B).

*Palb in combination with BA reduces cancer cell viability*. To determine whether ACLY inhibition could block the Palb-induced activation of ACLY, cells were treated with BA, Palb or their combination and evaluated the status of ACLY by western blotting. In both cell types, it was observed that BA reduced the activation of Palb-induced stimulation of ACLY (Fig. 2). To further investigate the effect of inhibiting the Palb-mediated ACLY activation in cancer cells, time course experiments of cell viability measurement using MDA-MB-231 and Panc1 cells were performed. Fig. 3A and B shows that cells treated with Palb and BA were significantly less viable that cells treated with either treatment alone.

The analysis was then expanded to include a panel of breast and pancreatic cancer cell lines. While Palb and BA had varied efficacies depending on the cell line, the combination of Palb and BA reduced the cell number to a greater extent compared with the effect on the cell number of either treatment alone in all cell types analyzed. Fig. 3C shows increased depletion of cell number in breast and pancreatic cell lines treated with Palb and BA compared with either treatment alone. These results were extended by using MDA-MB-231 cells grown in 3D culture. Cells allowed to form 3D structures more closely recapitulate the tumor environment and represent a good model of *in vivo* conditions (24). Cell viability and toxicity assays performed using MDA-MB-231 cells grown in 3D culture demonstrated that the combination of Palb and BA

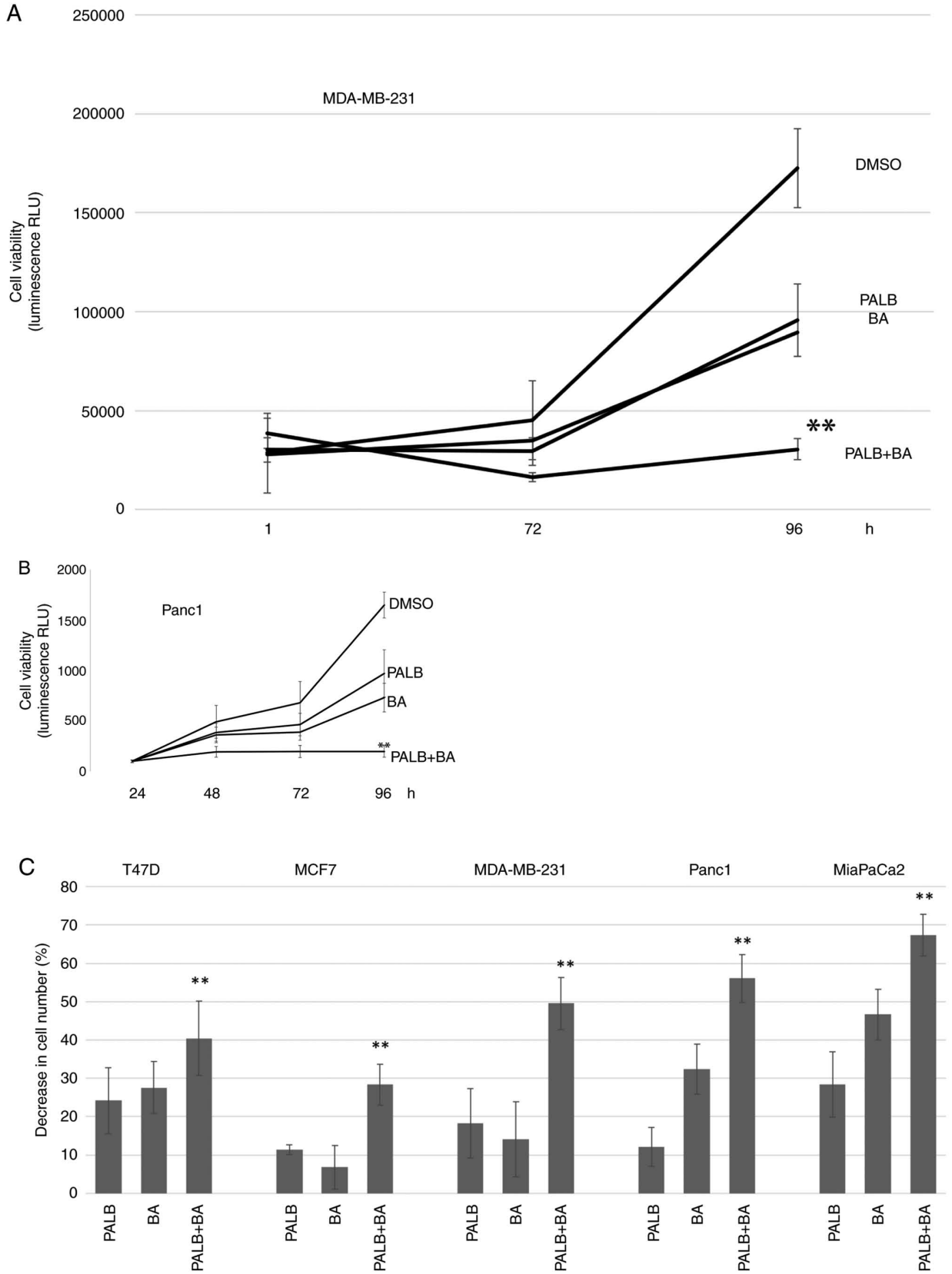


Figure 3. The combination of Palb and BA reduces cell viability further than either drug alone. (A) MDA-MB-231 or (B) Panc1 cells were treated with 0.1% DMSO, 1 $\mu$M Palb, 25 $\mu$M BA or the combination of Palb + BA for 96 h. Cell viability was determined in MDA-MB-231 cells at 1, 72 and 96 h and in Panc1 cells at 24, 48, 72 and 96 h after drug addition, using the RealTime-Glo MT Cell Viability assay that measures luminescence or RLU. Experiments were repeated twice with n=6 Error bars represent standard deviation of the mean. One-way ANOVA (of 96-h values) with Tukey's post hoc test was used to determine statistical significance. $^{**}$P<0.01 compared with Palb or BA treatment alone. (C) A panel of breast and pancreatic cancer cell lines were used in cell viability experiments. The CellTiter-Fluor Cell Viability Assay was used on cells after treatment with 0.1% DMSO, 1 $\mu$M P or 25 $\mu$M BA or the combination P + BA for 96 h. The average percent decrease in cell number compared with controls is shown and error bars represent standard deviation of the mean. Experiments were performed on each cell type three times with n=6. $^{**}$P<0.01 compared with Palb or BA treatment alone. Palb, palbociclib; BA, bempedoic acid; RLU, relative light units.

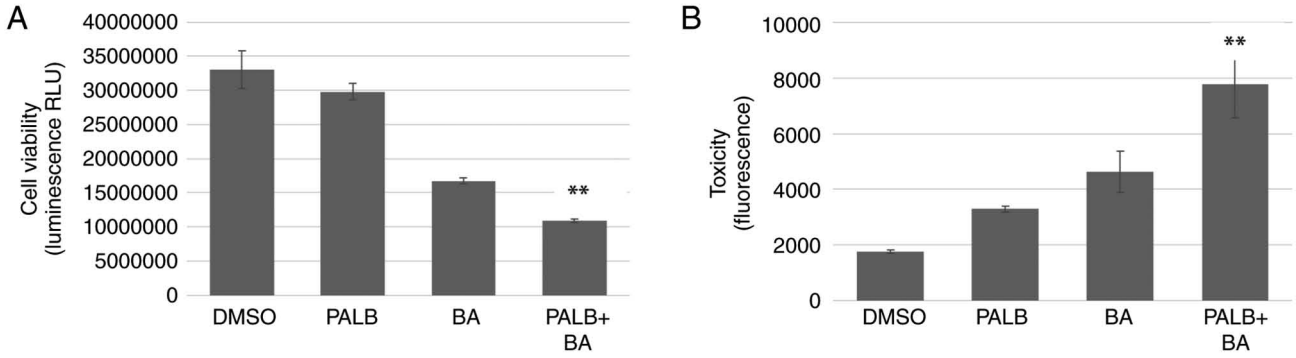


Figure 4. The combination of Palb and BA reduces viability of breast cancer cells grown in 3D cell culture. MDA-MB-231 cells were grown in 3D cell culture and treated with 0.1% DMSO, Palb (1 $\mu$M), BA (25 $\mu$M) or the combination P + BA for 96 h. (A) Cells in Matrigel formed spheres in 7 days and then were subjected to treatments. Cell viability was measured using CellTiter-Glo 3D Cell Viability Assay. (B) Cells were plated in ultra-low attachment plates that facilitate 3D sphere formation. Cell toxicity after treatment was measured using the CellTox Green Cytotoxicity Assay using Fluorescence (Ex:485 nm Em:520 nm). Experiments were repeated twice with n=8. Error bars represent standard deviation of the mean. One-way ANOVA with Tukey's post hoc test was used to determine statistical significance $^{**}$P<0.01 compared with Palb or BA treatment alone. Palb, palbociclib; BA, bempedoic acid.

reduced cell viability and increased toxicity compared with either treatment alone (Fig. 4).

*Palb inhibits proliferation, while BA induces apoptosis*. To determine the mechanism of action of Palb and BA in these experiments, proliferation and apoptosis in cancer cells treated with Palb or BA were measured. As shown in Fig. 5A, BrdU incorporation assays indicated that Palb inhibited proliferation while BA had no effect in both Panc1 and MDA-MBA-231 cells. Conversely, Annexin V assays revealed that BA induced apoptosis whereas Palb did not (Fig. 5B). To verify the target specificity of BA, the catalytic product of the ACLY enzyme, acetyl-CoA, was shown to block toxicity induced by BA in MDA-MBA-231 cells (Fig. 5C). Finally, the expression of markers of proliferation and apoptosis were evaluated by western blotting after cells were treated with either Palb or BA. In agreement with the results shown in Fig. 5A and B, cyclin D3 expression was reduced by Palb. In addition, the proliferation markers Mcm7 and PCNA showed reduced expression in response to Palb, but were unaffected by BA. After BA treatment, the markers of apoptosis cPARP and p-c-Jun were induced; however, cPARP was not induced by Palb (Fig. 5D).

*ACLY inhibition reduces cell invasion*. Since ACLY activity is required for fatty acid synthesis, and fatty acid synthesis is needed for cell membrane reorganization that occurs in EMT/invasion (25), we hypothesized that ACLY inhibition may block cancer cell invasion. To evaluate the effect of BA on cancer cell invasion, the criteria published in 2020 establishing that conclusions regarding EMT and invasiveness can only be confirmed by the existence of changes in expression of molecular markers in addition to changes in cellular properties were followed (26). Thus, two separate methods were utilized in the present analysis to evaluate EMT, invasion assays and western blotting detection of epithelial or mesenchymal markers. Firstly, the highly invasive fibrosarcoma cell line HT1080 was used. Transwell invasion assays using FBS as a chemoattractant revealed that treatment of these cells with BA resulted in significant inhibition of invasion compared with DMSO control and a decrease in the expression of the mesenchymal markers Snail by 69% and Slug by 70% compared with DMSO control (Fig. 6A). Similarly, ACLY inhibition in Panc1 cells blocked invasion and a decrease in the expression of the mesenchymal markers ZEB1 by 67% and Snail by 80% compared with control was observed (Fig. 6B). Finally, the MDA-MB-231 breast cancer cell line exhibited an increase in invasion when treated with 1 mM Palb that inhibits Rb phosphorylation in these cells (Figs. 1 and 2) accompanied by increased expression of the mesenchymal markers N-cadherin (76%), Vimentin (67%), ZEB1 (52%) and Slug (77%) when compared with DMSO controls (Fig. 6C). E-cadherin, an epithelial cell marker, exhibited a 51% increase in expression in response to BA treatment. Thus, BA treatment reversed the Palb-induced stimulation of invasion, and ACLY may be a reasonable target in strategies aimed at reversing AKT-mediated treatment resistance.

## Discussion

Over the last few decades, advances in the understanding of the crucial molecular mechanisms responsible for cancer cell signaling have elucidated the importance of the development of small molecule kinase inhibitors to target specific oncoproteins that fuel tumorigenesis (27). Kinase inhibition is a useful tool to specifically focus on a particular biochemical abnormality. Thus, as of 2022, there are 68 FDA approved kinase inhibitors used clinically to treat several types of neoplastic disease (27).

As efficacious as kinase inhibitors as a class have been, a common theme among patients with cancer treated with small-molecule inhibitors is the development of drug resistance. There are two mechanisms through which resistance occurs. Lack of treatment response to therapy is referred to as primary resistance, whereas resistance that occurs after an initial response to therapy and while still in treatment is known as acquired resistance. In the case of acquired resistance, tumors are thought to develop alternate mechanisms to evade the efficient blockade of cancer progression (28). The development of acquired drug resistance remains a major limitation and threat to the successful management of advanced cancer, and acquired resistance to the new class of CDK4/6 inhibitors is a significant clinical hurdle (5). These kinase

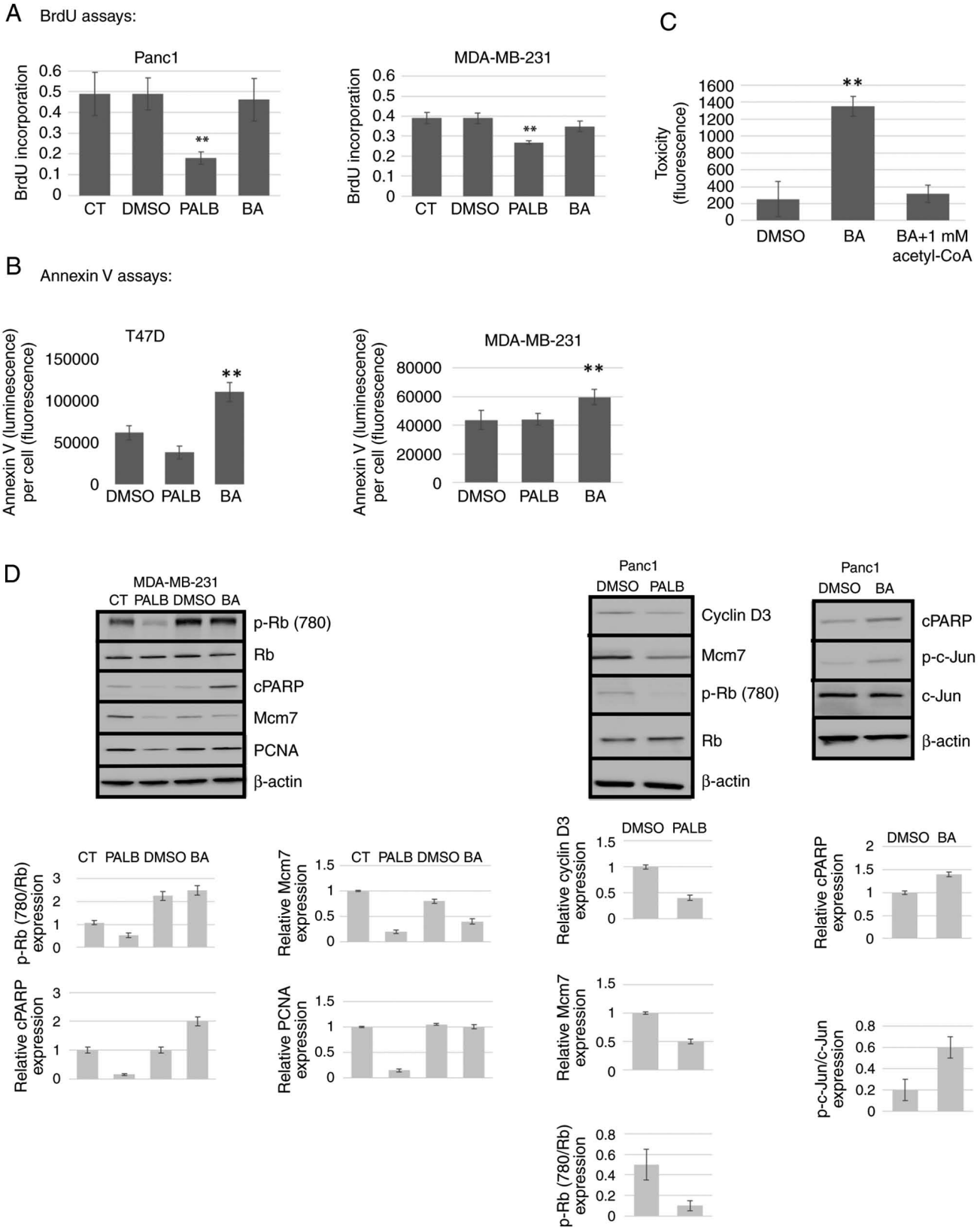


Figure 5. Palb inhibits proliferation and BA induces apoptosis. MDA-MB-231, Panc1 and T47D cells were used to analyze the mechanistic effects of P (1 $\mu$M) or BA (25 $\mu$M). Culture medium or 0.1% D were used as controls. (A) The BrdU cell proliferation assay was performed after drug treatments for 72 h. (B) The RealTime-Glo Annexin V assay was utilized to measure apoptosis 48 h after addition of drug and presented as Annexin V (absorbance)/cell number (fluorescence). Cell number was determined using the CellTiter-Fluor Cell Viability Assay. (C) Target validation of BA. The product of the ACLY enzyme (acetyl-CoA) was shown to reverse toxicity induced by BA using the CellTox Green Cytotoxicity Assay. Error bars display standard deviation of the mean of n=8. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. $^{**}$P<0.01 compared with all other treatments. (D) Western blotting was performed on cells treated for 96 h as aforementioned using antibodies to p-Rb (780), total Rb, apoptosis markers cPARP and p-c-Jun and the proliferation markers Mcm7, cyclin D3 and PCNA. β-actin was used as a loading control. Results shown were repeated twice. CT, control; p, phosphorylated; cPARP, cleaved poly (ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; BA, bempedoic acid; Palb, palbociclib; ACLY, ATP citrate lyase; BrdU, 5-bromo-2-deoxyuridine; Rb, retinoblastoma; Mcm7, minichromosome maintenance complex component 7.

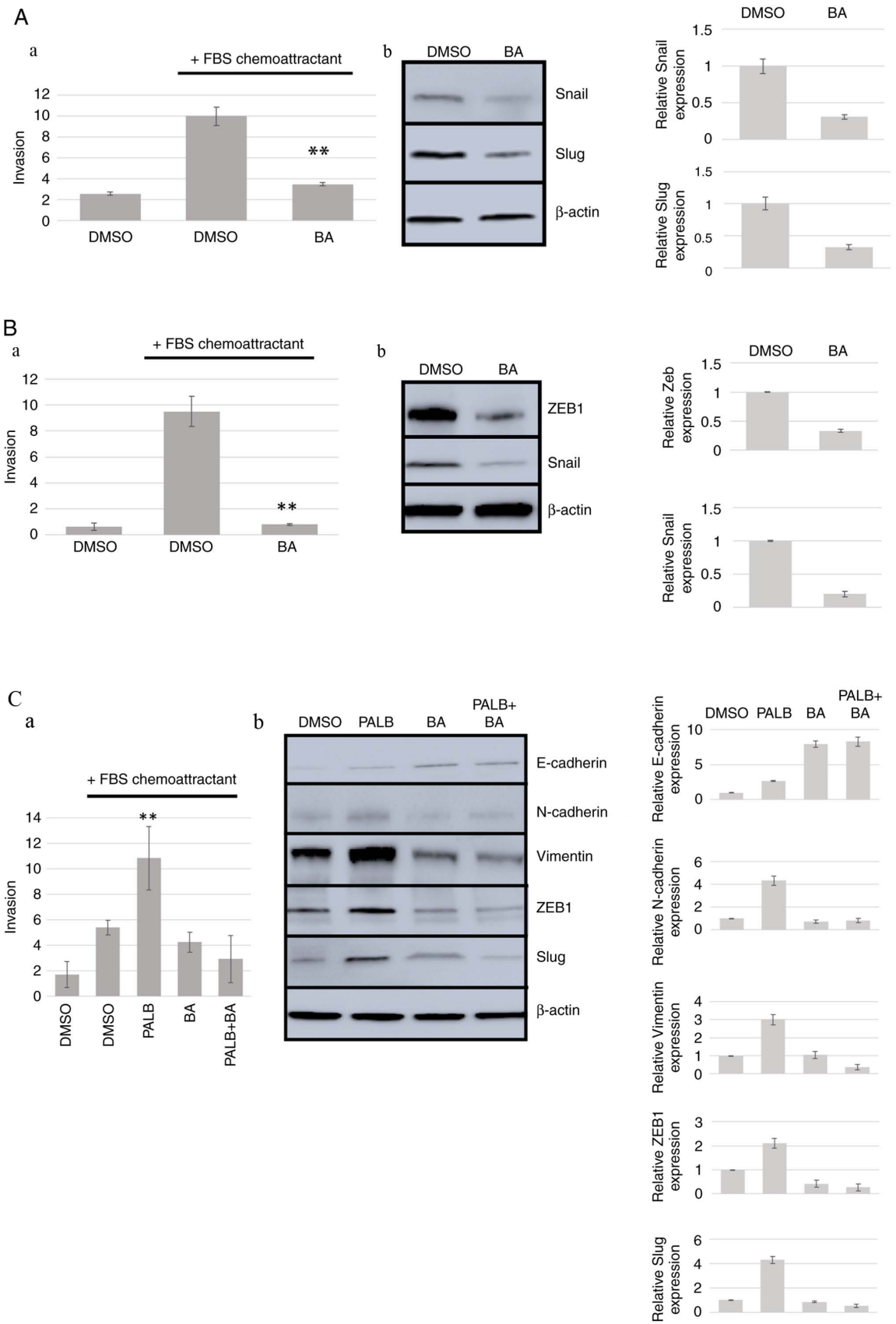


Figure 6. BA inhibits invasion in various cancer cell types. (Aa) HT1080 cells were treated with 0.1% DMSO or 25 $\mu$M BA, followed by assays as described in the Materials and methods. Statistical analysis was performed using unpaired Student's t-test. $^{**}$P<0.01 compared with DMSO control in the presence of FBS. (Ab) Detection of the mesenchymal markers Snail and Slug by western blotting. Results shown were repeated twice. (Ba) Panc1 cells were treated with 0.1% DMSO or 25 $\mu$M BA, followed by assays as described in the Materials and methods. Statistics were performed as in Aa. (Bb) Detection of the mesenchymal markers Snail and ZEB1 through western blotting. (Ca) MDA-MB-231 cells were treated with 0.1% D or 1 $\mu$M P for 96 h followed by invasion assays. One-way ANOVA with Tukey's post hoc test was used for statistical analysis. $^{**}$P<0.01 compared with all other treatments. Western blotting analysis was performed in (Cb) using antibodies against markers of the epithelial phenotype (E-cadherin) or the mesenchymal phenotype (N-cadherin, Vimentin, ZEB1 and Slug). β-actin was used as a loading control. Results shown were repeated twice. ZEB1, zinc finger E-box-binding homeobox 1; BA, bempedoic acid; Palb, palbociclib.

inhibitors were developed to target phosphorylation of Rb, as its inactivation by phosphorylation drives cell cycle progression. Currently, CDK4/6 inhibitors are widely utilized to treat estrogen receptor-positive, HER2-negative breast cancer in combination with hormone therapies, leading to doubling of the progression free survival compared with hormone therapy plus placebo. In addition, clinical trials testing the use of CDK4/6 inhibitors in several other cancer cell types (lung, pancreatic, colorectal, prostate, glioblastoma and squamous cell carcinoma of the head and neck) are presently underway (clinicaltrials.gov).

While CDK4/6 inhibition can have favorable effects in the clinic, most patients will experience disease progression while on treatment, probably through activation of alternate growth promoting pathways to acquire resistance (3). Preclinical studies have indicated that resistance to CDK4/6 inhibition may involve various pathways, one of which is the PI3K/AKT/mTOR pathway (4). This pathway mediates tumorigenic processes, such as increased proliferation and alterations in metabolism (29) Reprogrammed metabolism is considered a hallmark of cancer (30). Under aerobic conditions, normal cells convert glucose to pyruvate during glycolysis in the cytosol, and pyruvate is further metabolized in the mitochondria to generate most of the cellular ATP.

Several oncogenes have been implicated in stimulating glycolysis in tumor cells (29,31). For example, activation of the PI3K/AKT/mTOR pathway is a major contributor to carcinogenesis, both as a driving factor and as a mediator of resistance. AKT exerts a diverse collection of biological effects due to its varied substrates. As such, AKT can promote cell growth, survival, proliferation and alteration of metabolic processes that confer an advantage in tumorigenesis. In fact, the AKT signaling network interconnects oncogenic activity and cancer metabolism (29), as it stimulates glucose transport at the plasma membrane to enhance glucose uptake and glycolysis (32,33). Thus, AKT and glycolysis collaborate to stimulate the biosynthesis of lipids that are needed for cell growth and proliferation.

The class of biomolecules called lipids are derived from fatty acids, and include sterols, mono-, di- and triglycerides, phospholipids and glycolipids. While somatic cells obtain lipids from diet or from synthesis in the liver, cancer cells require an abundance of fatty acids for rapid production of the cell membrane (34). Lipid metabolism is often altered in cancer cells, such that *de novo* lipogenesis is stimulated making tumor cells impervious to externally available lipids (35). Increased lipogenesis has been shown to be required for tumor growth (34,36). Furthermore, *de novo* fatty acid synthesis has been associated with resistance to chemotherapies (37). In addition, the metabolic reprogramming of lipids is a newly recognized mechanism of resistance in breast cancer (20).

In cancer cells, cytoplasmic citrate is cleaved into acetyl-CoA and oxaloacetate by ACLY (38). Acetyl-CoA is the precursor for the synthesis of fatty acids, cholesterol and isoprenoids. Not only does AKT activation result in increased glycolysis and cytosolic citrate production, AKT directly activates ACLY by phosphorylation to promote fatty acid synthesis in cancer cells (16,39). Recently ACLY has been identified as a target with great therapeutic potential in cancer treatment (17). One of the early studies that predicted the importance of lipid metabolism in cancer demonstrated that ACLY exhibited a 160-fold increase in activity in breast cancer tumors compared with normal cells (40). Abnormally high levels of ACLY activity have been observed and are correlated with poor prognosis in several tumor types, such as non-small cell lung, colorectal, renal, ovarian, prostate, bladder and hepatocellular carcinoma and glioblastoma (17). Various cancer cell types, such as A549 lung adenocarcinoma, MCF7 breast cancer, LNCaP and DU145 prostate adenocarcinoma cells depend on ACLY activity for proliferation (18,19,39,41). Thus, we hypothesized that by inhibition of ACLY it may be possible to reverse the resistance pathway stimulated by AKT in response to CDK4/6 inhibition. The present study examined the effects of the combined inhibition of CDK4/6 and ACLY on cell proliferation, apoptosis and invasiveness of several cancer cell types.

The data described in the present study showed that CDK4/6 inhibition in cancer cells using Palb caused an increase in the activation of ACLY, which was mediated by AKT. To the best of our knowledge, this is the first study showing that CDK4/6 inhibition can lead to activation of this enzyme, and therefore may be involved in the altered metabolism observed in cells with reduced CDK4/6 activity (7,42). Increased ACLY activity due to CDK4/6 inhibition was reversed using BA. Furthermore, the combination of CDK4/6 inhibition with BA reduced cell viability more efficaciously than either treatment alone. This finding applied to several breast and pancreatic cancer cell types and was observed in breast cancer cells grown in 3D culture. The reduced cell number was shown to be due to CDK4/6 inhibition reducing proliferation, while ACLY inhibition using BA caused apoptosis. The combination of reduced proliferation with increased cell death accounts for the observed decrease in cell number and viability. It should be noted that these results may be explained in part by the fact that inhibition of synthesis of acetyl-CoA will alter the metabolic state of the cell and affect several processes, such as inflammation, histone acetylation and gene expression (43,44).

In order to evaluate the invasiveness of cancer cells treated with a combination of Palb and BA, EMT was analyzed. In the present study, an increase in the expression of EMT-transcription factors (TFs) N-cadherin, ZEB1, Vimentin and Slug in breast cancer cells in response to Palb was observed, suggesting that CDK4/6 inhibition may affect EMT. These experiments were undertaken due to the conflicting available information regarding the effect of CDK4/6 inhibition on invasion. The effect of CDK4/6 inhibition generally and Palb specifically on cancer cell invasiveness and metastasis is unclear. Interestingly, the phase 3 PALbociclib CoLlaborative Adjuvant Study (PALLAS) clinical trial measured invasive disease-free survival in patients with estrogen receptor-positive, HER2-negative breast cancer treated with Palb in addition to standard adjuvant endocrine therapy. It was reported in early 2022 that Palb is unlikely to improve patient outcomes over endocrine therapy alone (45). With an endpoint of metastatic disease, Palb appears not to improve patient response. This finding elicits the question of how CDK4/6 inhibition affects cancer cell invasiveness. Preclinical studies, to date, are conflicting in their findings.

In studies that measure invasiveness or EMT in cancer cells treated with Palb, using short (<48 h) CDK4/6 inhibitor treatments leads to a decrease in EMT or invasiveness, while cell treatment for longer time periods (3-8 days) results in a stimulatory effect on invasiveness in response to Palb treatment (46-48). Interestingly, in a short 24-h Palb treatment of pancreatic cancer cells, Palb caused inhibition of EMT; however, this duration of treatment did not lead to activation of the AKT pathway (47). Similarly, in MDA-MB-231 breast cancer cells a 48-h treatment of Palb caused a decrease in ZEB1 protein and inhibition of invasion (48). However, as observed in the present study using MDA-MB-231 breast cancer cells, longer treatment with Palb caused increased expression of the mesenchymal transcription factor ZEB1 and increased invasiveness.

In agreement with the current results, a study using treatments that lasted between 72 h and 8 days in pancreatic cancer cells showed a significant increase in invasion and EMT (46). Thus, it may be that Palb treatment of a sufficiently longer duration leads to increased invasiveness, which may be a contributor to Palb-mediated acquired resistance. To test the effect of ACLY inhibition on cancer cells, HT1080 cells, a highly invasive fibrosarcoma cell line, was used. It was observed that ACLY inhibition reduced the invasiveness of HT1080 cells, as well as that of the pancreatic cell line Panc1. In MDA-MB-231 breast cancer cells, invasion was stimulated by CDK4/6 inhibition, whereas ACLY inhibition reversed the increase in the observed invasiveness.

Interestingly, EMT has been implicated as a factor in the mechanism of resistance. The loss of E-cadherin has been associated with resistance to growth factor and kinase inhibition. In non-small cell lung cancer, mesenchymal cell lines (defined as expressing N-cadherin and Vimentin) exhibited marked resistance to targeted therapies to the EGFR and the PI3K/AKT pathways compared with that of epithelial cell lines (expressing high levels of E-cadherin) (49). The mechanism of resistance is postulated to occur via EMT-TF-mediated suppression of apoptosis (50). Thus, the present results cannot rule out the possibility that an increase in the expression of EMT-TFs in breast cancer in response to CDK4/6 inhibition may contribute to acquired resistance. As BA is a prodrug requiring activation by acyl-CoA synthetase very long chain family member 1, it is essential to note that this enzyme is expressed in breast and pancreatic cancer cells (51,52). In addition, although both Palb and BA are currently utilized to treat patients with cancer or high cholesterol, the combination of these treatments has not been tested in animal models to date, to the best of our knowledge. Thus, additional studies are needed to further elucidate the usefulness and safety of the combination strategy utilized in the present study in the clinical setting.

**Acknowledgements**

Not applicable.

**Funding**

The present work was supported by the National Cancer Institute of the National Institutes of Health (grant no. R15CA231372).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

NK initiated the work, performed the apoptosis and cell proliferation experiments, analyzed data and wrote the manuscript with BV and CP. BV, KC and RK designed, performed and analyzed the cell viability experiments. KD designed, performed and analyzed the AKT knockdown and ACLY activation by western blotting. CP and DK designed, performed and analyzed the invasion experiments (Transwell assays and western blotting). NK and BV confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Mittnacht S: The retinoblastoma protein-from bench to bedside. Eur J Cell Biol 84: 97-107, 2005.
2. Asghar U, Witkiewicz AK, Turner NC and Knudsen ES: The history and future of targeting cyclin-dependent kinases in cancer therapy. Nat Rev Drug Discov 14: 130-146, 2015.
3. Guarducci C, Bonechi M, Boccalini G, Benelli M, Risi E, Di Leo A, Malorni L and Migliaccio I: Mechanisms of resistance to CDK4/6 inhibitors in breast cancer and potential biomarkers of response. Breast Care (Basel) 12: 304-308, 2017.
4. Hamilton E and Infante JR: Targeting CDK4/6 in patients with cancer. Cancer Treat Rev 45: 129-138, 2016.
5. McCartney A, Migliaccio I, Bonechi M, Biagioni C, Romagnoli D, De Luca F, Galardi F, Risi E, De Santo I, Benelli M, *et al*: Mechanisms of resistance to CDK4/6 inhibitors: Potential implications and biomarkers for clinical practice. Front Oncol 9: 666, 2019.
6. Guerrero-Zotano A, Mayer IA and Arteaga CL: PI3K/AKT/mTOR: Role in breast cancer progression, drug resistance, and treatment. Cancer Metastasis Rev 35: 515-524, 2016.
7. Franco J, Balaji U, Freinkman E, Witkiewicz AK and Knudsen ES: Metabolic reprogramming of pancreatic cancer mediated by CDK4/6 inhibition elicits unique vulnerabilities. Cell Rep 14: 979-990, 2016.
8. Zhang J, Xu K, Liu P, Geng Y, Wang B, Gan W, Guo J, Wu F, Chin YR, Berrios C, *et al*: Inhibition of Rb phosphorylation leads to mTORC2-mediated activation of Akt. Mol Cell 62: 929-942, 2016.
9. Jansen VM, Bhola NE, Bauer JA, Formisano L, Lee KM, Hutchinson KE, Witkiewicz AK, Moore PD, Estrada MV, Sánchez V, *et al*: Kinome-wide RNA interference screen reveals a role for PDK1 in acquired resistance to CDK4/6 inhibition in ER-positive breast cancer. Cancer Res 77: 2488-2499, 2017.
10. Litchfield LM, Boehnke K, Brahmachary M, Mur C, Bi C, Stephens JR, Sauder JM, Gutiérrez SM, McNulty AM, Ye XS, *et al*: Combined inhibition of PIM and CDK4/6 suppresses both mTOR signaling and Rb phosphorylation and potentiates PI3K inhibition in cancer cells. Oncotarget 11: 1478-1492, 2020.

11. Herrera-Abreu MT, Palafox M, Asghar U, Rivas MA, Cutts RJ, Garcia-Murillas I, Pearson A, Guzman M, Rodriguez O, Grueso J, *et al*: Early adaptation and acquired resistance to CDK4/6 inhibition in estrogen receptor-positive breast cancer. Cancer Res 76: 2301-2313, 2016.
12. Franco J, Witkiewicz AK and Knudsen ES: CDK4/6 inhibitors have potent activity in combination with pathway selective therapeutic agents in models of pancreatic cancer. Oncotarget 5: 6512-6525, 2014.
13. Vora SR, Juric D, Kim N, Mino-Kenudson M, Huynh T, Costa C, Lockerman EL, Pollack SF, Liu M, Li X, *et al*: CDK 4/6 inhibitors sensitize PIK3CA mutant breast cancer to PI3K inhibitors. Cancer Cell 26: 136-149, 2014.
14. LoRusso PM: Inhibition of the PI3K/AKT/mTOR pathway in solid tumors. J Clin Oncol 34: 3803-3815, 2016.
15. Xu W, Yang Z and Lu N: A new role for the PI3K/Akt signaling pathway in the epithelial-mesenchymal transition. Cell Adh Migr 9: 317-324, 2015.
16. Berwick DC, Hers I, Heesom KJ, Moule SK and Tavare JM: The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. J Biol Chem 277: 33895-33900, 2002.
17. Granchi C: ATP citrate lyase (ACLY) inhibitors: An anti-cancer strategy at the crossroads of glucose and lipid metabolism. Eur J Med Chem 157: 1276-1291, 2018.
18. Hatzivassiliou G, Zhao F, Bauer DE, Andreadis C, Shaw AN, Dhanak D, Hingorani SR, Tuveson DA and Thompson CB: ATP citrate lyase inhibition can suppress tumor cell growth. Cancer Cell 8: 311-321, 2005.
19. Migita T, Okabe S, Ikeda K, Igarashi S, Sugawara S, Tomida A, Taguchi R, Soga T and Seimiya H: Inhibition of ATP citrate lyase induces an anticancer effect via reactive oxygen species: AMPK as a predictive biomarker for therapeutic impact. Am J Pathol 182: 1800-1810, 2013.
20. Feng WW and Kurokawa M: Lipid metabolic reprogramming as an emerging mechanism of resistance to kinase inhibitors in breast cancer. Cancer Drug Resist 3: 1-17, 2020.
21. Ray KK, Bays HE, Catapano AL, Lalwani ND, Bloedon LT, Sterling LR, Robinson PL and Ballantyne CM; CLEAR Harmony Trial: Safety and efficacy of bempedoic acid to reduce LDL cholesterol. N Engl J Med 380: 1022-1032, 2019.
22. Debnath J, Muthuswamy SK and Brugge JS: Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30: 256-268, 2003.
23. Egger JV, Lane MV, Antonucci LA, Dedi B and Krucher NA: Dephosphorylation of the retinoblastoma protein (Rb) inhibits cancer cell EMT via Zeb. Cancer Biol Ther 17: 1197-1205, 2016.
24. Breslin S and O'Driscoll L: The relevance of using 3D cell cultures, in addition to 2D monolayer cultures, when evaluating breast cancer drug sensitivity and resistance. Oncotarget 7: 45745-45756, 2016.
25. Luo X, Cheng C, Tan Z, Li N, Tang M, Yang L and Cao Y: Emerging roles of lipid metabolism in cancer metastasis. Mol Cancer 16: 76, 2017.
26. Yang J, Antin P, Berx G, Blanpain C, Brabletz T, Bronner M, Campbell K, Cano A, Casanova J, Christofori G, *et al*: Guidelines and definitions for research on epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 21: 341-352, 2020.
27. Roskoski R Jr: Properties of FDA-approved small molecule protein kinase inhibitors: A 2022 update. Pharmacol Res 175: 106037, 2022.
28. Lovly CM and Shaw AT: Molecular pathways: Resistance to kinase inhibitors and implications for therapeutic strategies. Clin Cancer Res 20: 2249-2256, 2014.
29. Hoxhaj G and Manning BD: The PI3K-AKT network at the interface of oncogenic signalling and cancer metabolism. Nat Rev Cancer 20: 74-88, 2020.
30. Hanahan D: Hallmarks of cancer: New dimensions. Cancer Discov 12: 31-46, 2022.
31. DeBerardinis RJ and Chandel NS: Fundamentals of cancer metabolism. Sci Adv 2: e1600200, 2016.
32. Kohn AD, Summers SA, Birnbaum MJ and Roth RA: Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 271: 31372-31378, 1996.
33. Roberts DJ, Tan-Sah VP, Smith JM and Miyamoto S: Akt phosphorylates HK-II at Thr-473 and increases mitochondrial HK-II association to protect cardiomyocytes. J Biol Chem 288: 23798-23806, 2013.
34. Currie E, Schulze A, Zechner R, Walther TC and Farese RV Jr: Cellular fatty acid metabolism and cancer. Cell Metab 18: 153-161, 2013.
35. Menendez JA and Lupu R: Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer 7: 763-777, 2007.
36. Santos CR and Schulze A: Lipid metabolism in cancer. FEBS J 279: 2610-2623, 2012.
37. Rysman E, Brusselmans K, Scheys K, Timmermans L, Derua R, Munck S, Van Veldhoven PP, Waltregny D, Daniëls VW, Machiels J, *et al*: De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. Cancer Res 70: 8117-8126, 2010.
38. Srere PA: The citrate cleavage enzyme. I. Distribution and purification. J Biol Chem 234: 2544-2547, 1959.
39. Bauer DE, Hatzivassiliou G, Zhao F, Andreadis C and Thompson CB: ATP citrate lyase is an important component of cell growth and transformation. Oncogene 24: 6314-6322, 2005.
40. Szutowicz A, Kwiatkowski J and Angielski S: Lipogenetic and glycolytic enzyme activities in carcinoma and nonmalignant diseases of the human breast. Br J Cancer 39: 681-687, 1979.
41. Zhang C, Liu J, Huang G, Zhao Y, Yue X, Wu H, Li J, Zhu J, Shen Z, Haffty BG, *et al*: Cullin3-KLHL25 ubiquitin ligase targets ACLY for degradation to inhibit lipid synthesis and tumor progression. Genes Dev 30: 1956-1970, 2016.
42. Lorito N, Bacci M, Smiriglia A, Mannelli M, Parri M, Comito G, Ippolito L, Giannoni E, Bonechi M, Benelli M, *et al*: Glucose metabolic reprogramming of ER breast cancer in acquired resistance to the CDK4/6 inhibitor palbociclib. Cells 9: 668, 2020.
43. Santarsiero A, Convertini P, Todisco S, Pierri CL, De Grassi A, Williams NC, Iacobazzi D, De Stefano G, O'Neill LAJ and Infantino V: ACLY nuclear translocation in human macrophages drives proinflammatory gene expression by NF-κB acetylation. Cells 10: 2962, 2021.
44. Shi L and Tu BP: Acetyl-CoA and the regulation of metabolism: Mechanisms and consequences. Curr Opin Cell Biol 33: 125-131, 2015.
45. Gnant M, Dueck AC, Frantal S, Martin M, Burstein HJ, Greil R, Fox P, Wolff AC, Chan A, Winer EP, *et al*: Adjuvant palbociclib for early breast cancer: The PALLAS trial results (ABCSG-42/AFT-05/BIG-14-03). J Clin Oncol 40: 282-293, 2022.
46. Liu F and Korc M: Cdk4/6 inhibition induces epithelial-mesenchymal transition and enhances invasiveness in pancreatic cancer cells. Mol Cancer Ther 11: 2138-2148, 2012.
47. Rencuzogulları O, Yerlikaya PO, Gürkan AÇ, Arısan ED and Telci D: Palbociclib, a selective CDK4/6 inhibitor, restricts cell survival and epithelial-mesenchymal transition in Panc-1 and MiaPaCa-2 pancreatic cancer cells. J Cell Biochem 121: 508-523, 2020.
48. Zhang Z, Li J, Ou Y, Yang G, Deng K, Wang Q, Wang Z, Wang W, Zhang Q, Wang H, *et al*: CDK4/6 inhibition blocks cancer metastasis through a USP51-ZEB1-dependent deubiquitination mechanism. Signal Transduct Target Ther 5: 25, 2020.
49. Witta SE, Gemmill RM, Hirsch FR, Coldren CD, Hedman K, Ravdel L, Helfrich B, Dziadziuszko R, Chan DC, Sugita M, *et al*: Restoring E-cadherin expression increases sensitivity to epidermal growth factor receptor inhibitors in lung cancer cell lines. Cancer Res 66: 944-950, 2006.
50. Du B and Shim JS: Targeting epithelial-mesenchymal transition (EMT) to overcome drug resistance in cancer. Molecules 21: 965, 2016.
51. Monaco ME: Fatty acid metabolism in breast cancer subtypes. Oncotarget 8: 29487-29500, 2017.
52. Hansel DE, Rahman A, House M, Ashfaq R, Berg K, Yeo CJ and Maitra A: Met proto-oncogene and insulin-like growth factor binding protein 3 overexpression correlates with metastatic ability in well-differentiated pancreatic endocrine neoplasms. Clin Cancer Res 10: 6152-6158, 2004.