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參展科別　醫學與健康科學

作品名稱　**PP5-AMPK Pathway mediates Palbociclib-induced Cell Death in Lung Cancer**

得獎獎項　大會獎：一等獎

青少年科學獎

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關鍵詞　**Lung Cancer、Palbociclib、AMPK**

作者簡介

我是陳禹縉，台北市私立復興中學雙語部高二學生。從小便對科學展現濃烈興趣，喜歡觀察與凡事動手做。很高興近年能有機會進入實驗室做研究。實驗室內有數不清的失敗與氣餒，這些跌跌撞撞的時刻，教會了我凡事永不言棄。期望有一天，我能憑藉著在科學上的熱力與發現，真正為有需要的人提供幫助。

研究摘要

肺癌是目前全世界首要致死的癌症。雖然現有的標靶治療與免疫療法已經改善某些病患的情況，但有許多病患仍無有效治療方法。因此，我們急需探求新的藥物治療方法來改善肺癌治療的成效。

在此我們選擇一個新標靶藥物Palbociclib來進行研究，Palbociclib 是剛核准的乳癌藥物，主要是藉由抑制CDK4/6來延緩乳癌生長。我們發現Palbociclib能有效地抑制肺癌細胞株的生長和死亡。特別的是，Palbociclib不僅誘發肺癌細胞凋亡(apoptosis)，亦誘發肺癌細胞自噬死亡(Autophagy)。因為AMPK能同時影響細胞凋亡與自噬死亡，我們進一步研究AMPK在Palbociclib誘發癌細胞死亡中的角色，發現Palbociclib主要是藉由抑制去磷酸酶PP5的活性，來增加AMPK的磷酸化，進而活化AMPK，誘發肺癌細胞產生凋亡與自噬。這些藥效原非抑制CDK4/6會產生的作用，我們發現Palbociclib藉由調控PP5-AMPK路徑以誘發細胞死亡。希望這些研究結果能幫助肺癌的臨床研究，以造福病患。

Abstract

Lung cancer is the worldwide leading cause of cancer death currently. The majority of lung cancer patients are diagnosed with non-small-cell lung cancer (NSCLC). Advances have been made with targeted therapies against specific genetic aberrations and with the development of immunotherapies. However, there are still a huge portion of patients that do not respond to current medications. Therefore, one of the unmet needs in oncology is the identification and development of new treatment modalities against NSCLC. Palbociclib is a potent cyclin dependent kinase 4/6 (CDK4/6) inhibitor approved in advanced breast cancer. In the current study, we found that Palbociclib treatment reduced cell viability, inhibited colony formation and triggered cell death in NSCLC cell lines. Palbociclib-induced apoptosis and autophagy were observed. The upregulation of p-AMPK (Thr172) implied that Palbociclib enhanced the activity of AMPK. Inhibition of AMPK activity by compound c and AMPK silencing by exploiting siRNA against AMPK both relieved NSCLC cells from Palbociclib-induced apoptosis and autophagy. Reduced activity of protein phosphatase 5 (PP5), a serine/threonine phosphatase which negatively regulated the phosphorylation of AMPK, was observed in NSCLC cells incubated with Palbociclib, while the protein expression of PP5 remained unchanged. Activation of PP5 by employing a PP5 agonist arachidonic acid (AA), or ectopic expression of PP5, both reduced the phosphorylation of AMPK and rescued NSCLC cells from Palbociclib-triggered cell death. Our report described herein demonstrate that, aside from CDK4/6 inhibition, Palbociclib also suppressed PP5 activity and enhanced the phosphorylation of AMPK, leading to cancer cells growth inhibition in NSCLC. Hopefully, this study may provide a rationale for clinical assessments of Palbociclib’s effects against NSCLC.

一、前言

（一）研究動機

Lung cancer is the most common type of cancer globally. It ranks first as the leading cause of cancer death, and accounts for more than 1.7 million deaths worldwide in 2018. To date, the two main subtypes of lung cancers are small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), which is responsible for approximately 85% of lung cancers. NSCLC can be further histologically categorized into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Despite the fact that early stage NSCLCs can be removed by surgery, patients diagnosed with advanced NSCLCs are not expected to be cured by surgical management.

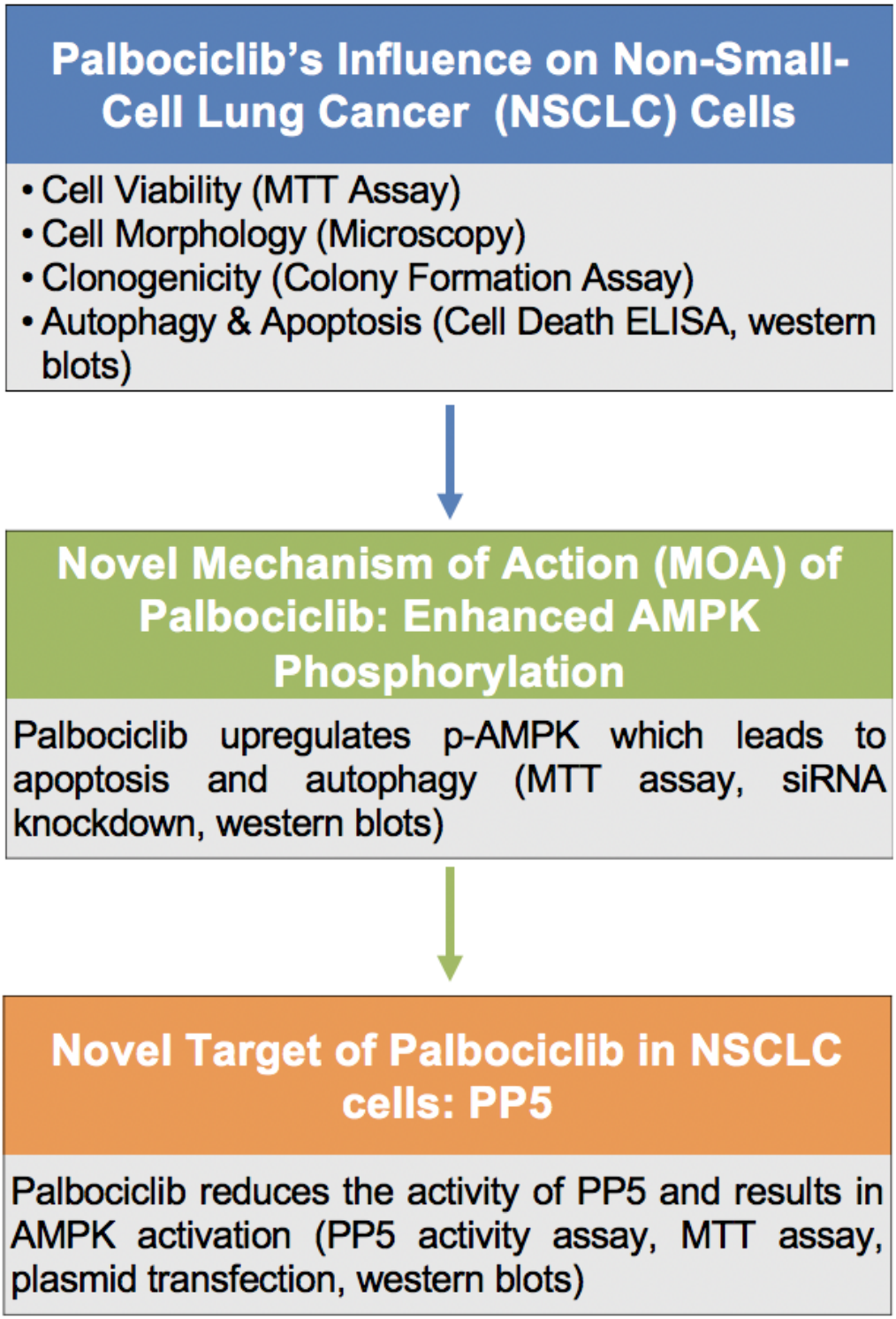
Chemotherapy, radiotherapy, targeted therapy, and immunotherapy are the main treatment modalities of NSCLC. For the past decade, the concept of precision medicine has evolved. Depending on the gene mutational status, targeted agents against EGFR (such as gefitinib, erlotinib, afatinib and osimertinib), ALK (such as crizotinib), and BRAF (such as dabrafenib) mutations have been developed [1]. While gaining initial success, drug resistance usually occurs and is one of the major causes for treatment failure. For advanced NSCLC patients lacking driver mutations, immune checkpoint inhibitors [2], such as pembrolizumab, nivolumab, and atezolizumab, have made significant progress for NSCLC management. However, only a portion of NSCLC patients (about 25%) respond to immune checkpoint inhibitors. Hence, the identification and development of new therapies against NSCLC are urgently desired.

In this study, we investigated a new drug called Palbociclib, a CDK 4/6 inhibitor, which has recently been approved for patients with advanced breast cancer. Palbociclib shows anti-cancer activity in several pre-clinical tumor models [3] besides breast cancer. However, its activity against NSCLC remains largely unknown. Therefore, we seek to investigate the effects of Palbociclib treatment on NSCLC cells and unveil the potential of Palbociclib to a broader spectrum of tumor therapy.

（二）研究目的

1. Test the effects of Palbociclib on lung cancer cells.
2. Explore the mode of actions of Palbociclib on lung cancer cells.

二、研究方法與過程


Palbociclib's Influence on Non-Small-Cell Lung Cancer (NSCLC) Cells
• Cell Viability (MTT Assay)
• Cell Morphology (Microscopy)
• Clonogenicity (Colony Formation Assay)
• Autophagy & Apoptosis (Cell Death ELISA, western blots)
Novel Mechanism of Action (MOA) of Palbociclib: Enhanced AMPK Phosphorylation
Palbociclib upregulates p-AMPK which leads to apoptosis and autophagy (MTT assay, siRNA knockdown, western blots)
Novel Target of Palbociclib in NSCLC cells: PP5
Palbociclib reduces the activity of PP5 and results in AMPK activation (PP5 activity assay, MTT assay, plasmid transfection, western blots)

（一）Reagents, chemicals, antibodies, and plasmid constructs

Palbociclib used for *in vitro* analyses was from MedKoo Biosciences (Morrisville, NC, USA). For *in vivo* studies, Palbociclib was from MedChem Express. Cell death ELISA kit was purchased from Roche (Roch e Life Science, Mannheim, Germany). 3-methyladenine (3-MA) was acquired from Cayman Chemical (A nn Arbor, MI, USA). Arachidonic acid (AA) was a product of BioVision (Milpitas, CA, USA). Z-VAD-F MK and Compound C (dorsomorphin dihydrochloride) were both purchased from MedChem Express (M onmouth Junction, NJ, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was f rom Sigma-Aldrich (St. Louis, MO, USA). DharmaFECT 4 transfection reagent, siAMPK α 2 (PRKAA2, #L-005361-00-0005) and siControl (#D-001810-10-50) were obtained from Dharmacon (Lafayette, CO, USA). pCMV-Taq2B-vector was purchased from Agilent Technologies (Santa Clara, CA, USA) to constr uct pCMV-Taq2B-PP5. Serine/Threonine Phosphatase Assay Kit used to measure the activity of PP5 was purchased from Thermo Fisher Scientific (Bridgewater, NJ, USA). Antibodies used in immunoblotting ex periments were indicated below. Mouse anti-actin antibody was obtained from Proteintech (Rosemont, IL, USA). Mouse anti-PARP-1, Goat anti-PP5 (C-20), Donkey anti-goat IgG-HRP, Goat anti-rabbit IgG-HR P, and Goat anti-mouse IgG-HRP were acquired from Santa Cruz Biotechnology (Dallas, TX, USA). Mo use anti-DDK antibody was from OriGene Technologies (Rockville, MD, USA). Other antibodies, such a s AMPK α , phospho-AMPK α (Thr172), ULK1, Phospho-ULK1 (Ser137), LC3B, Caspase 8, Caspase 9, and Caspase 3 were all purchased from Cell Signaling Technology (Danvers, MA, USA). RPMI-1640, fet al bovine serum (FBS), Opti-MEM, 10X phosphate buffered saline (PBS), Lipofectamine 2000 reagent, a nd 10X Trypsin-EDTA were purchased from Thermo Fisher Scientific (Bridgewater, NJ, USA). PSA (Pe nicillin, Streptomycin, and Amphotericin B solution) was obtained from Biological Industries (Cromwell, CT, USA).

（二） Cell culture

Human lung adenocarcinoma cell lines A549 and H358 were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Cells were cultured in RPMI medium containing 10% FBS. A549 and H358 cells were cultured in 100 mm cell culture dishes. When NSCLC cells reached 80% conf luency, cells were washed with PBS for 2 times to remove FBS, which may contain trypsin inhibitors. Af ter that, trypsin solution was added to the culture dish and incubated for 5 minutes at 37°C to detach the c ells. Cells, following detachment from the culture dish, were mixed with an equal volume of FBS-contain ing cell culture medium and transferred to a centrifuge tube. After that, the cell-containing tube was spun at 900 rpm for 5 minutes. Supernatant was then removed and cells were re-suspended in culture medium f

or cell count by using a hemocytometer. Cells were seeded at a density of 1 x10^4/cm^2 and cultured in a C O2 incubator.

（三） Cell viability

Cells were seeded at a density of 5000 cells/well in a 96-well plate. After attachment, cells were treated w ith Palbociclib at pre-determined concentrations for 24-72 hours. After that, 25 μl of 5X MTT solution w as added to the well to make a concentration of MTT at 1 mg/ml. The 96-well was incubated in the CO2 i ncubator for 3 hours. Next, supernatant was removed and 100 μl of DMSO was added to solubilize the p urple MTT crystals. The amounts of living cells per well was directly proportional to the intensity of the f ormazan dye (purple), and was assessed by measuring the absorbance at 570 nm using a plate reader.

（四）Cell death ELISA

Cell death ELISA was performed according to user’ s manual to examine the effects of Palbociclib on a poptotic cell death. In brief, NSCLC cells were cultured in the 96-well plate and treated with different co ncentrations of Palbociclib. Then, 200 μl lysis buffer was added to the wells, and the plate was incubated for 30 minutes at room temperature. Cells were collected and centrifuged at 200 g for 10 minutes. Next, 2 0 μl of lysates were added to each well to react with 80 μl of anti-Histone- and anti-DNA-peroxidase (PO D)-containing immunoreagent for 2 hours. Supernatant was washed before the addition of 100 μl substrat e solution. Then, 100 μl of stop solution was added and the detection of released cytoplasmic nucleosome s were performed by measuring the absorbance at 405 nm.

（五）Colony formation assay

Cells were seeded in a six-well plate at a density of 1000 cells/well. Cells were treated with Palbociclib at pre-defined concentrations for 14 days. Then, cell colonies are fixed with 6% glutaraldehyde and stained with 0.5% crystal violet solution for 30 minutes.

（六）Examination of PP5 activity

A549 and H358 cells were treated with 15 μM palbociclib for 24 hours. Then, cells were harvested and i mmunoprecipitation was performed using antibodies against PP5. In brief, NP-40 lysis buffer supplement ed with protease inhibitors was used for cell lysis. Then, protein quantification was performed and 750 μg of protein was incubated with 1 μg of antibodies against PP5 at 4°C overnight. After that, protein G beads were added and incubated for 3 hours. The beads were further washed with NP-40 buffer before the PP5-

containing cell lysates were collected. Cellular PP5 activity was further assessed by exploiting the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit.

For PP5 activity measurement in the cell-free condition, immunoprecipitation was first performed using antibodies against PP5 to collect PP5-containing A549 immunoprecipitants. Then, I incubated Palbociclib (1 and 10 μM) with the A549 immunoprecipitants for 30 minutes at room temperature. Serine/Threonine Phosphatase Assay was next conducted according to the user’ s manual for PP5 activity measurement. Fluorescence was measured at 465 nm.

（七）Western blot analyses

Cells were harvested and collected using modified RIPA lysis buffer (150mM NaCl, 1mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, and 50mM Tris-HCl pH 7.4,) freshly supplemented with 1mM PMSF, 1X protease inhibitor, 0.1% SDS, 1mM NaF, and 1mM Na_3VO_4. The lysates were incubated on ice for 30 minutes before being centrifuged at 13500 rpm for 30 minutes. The supernatants were collected for protein quantification. Equal amount of protein was subjected to SDS-PAGE for 120 minutes at 120V and then transferred to PVDF membranes. The membranes were immunoblotted for pre-determined primary antibodies at 4°C overnight. Then, the membranes were further probed with 2nd antibodies for 1 hour at room temperature. The signals were next detected using enhanced chemiluminescence (ECL) solution and X-ray films.

（八）Plasmid transfection

1 μg of either pCMV-Taq2B-PP5 or pCMV-Taq2B-vector was incubated with 150 μl of opti-MEM in tube 1 for 5 minutes. 3 μl of Lipofectamine 2000 was incubated with 150 μl opti-MEM in tube 2 for 5 minutes. The two tubes were mixed and incubated for 20 minutes. Then, the mixture was added to a well in the 6-well culture dish for 24 hours at 37°C. The wells were then washed for further experiments.

（九）Small interfering RNA (siRNA) transfection

5 μl of siRNA was incubated with 195 μl of opti-MEM in tube 1 for 5 minutes. 4 μl of DharmaFECT 4 transfection reagent and 196 μl of opti-MEM was incubated in tube 2 for 5 minutes. Then, tube 1 and tube 2 were mixed and incubated for 20 minutes. The mixture was then supplemented to a well containing 1600 μl of cell culture medium in the 6-well plate. The concentration of siRNA in a well was 50 nM. The 6-well plate was placed in the 37°C CO2 incubator for 24 hours before further experiments were conducted.

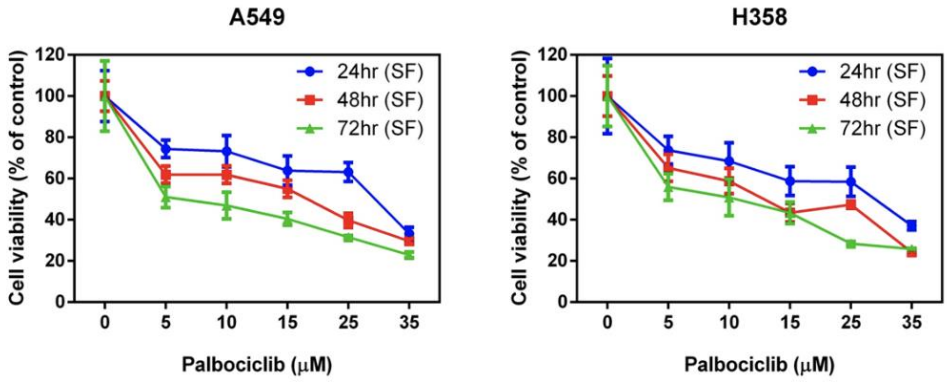
（十）Statistics

Values are shown as mean ± SD. Statistical analyses were done by two-way ANOVA or Student’ s *t*-test using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered t o be statistically significant.

三、研究結果與討論

（一）研究結果

Our initial approach was to see how Palbociclib treatment leads to the change in cell viability and morph ology in lung cancer cells. I treated NSCLC cell lines, H358 and A549, with different concentrations of P albociclib (0, 5, 10, 15, 25, 35 μM) for 24, 48 and 72 hours. Cell viability was assessed by performing MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. As shown in Figure 1, Palb ociclib treatment resulted into decreased cell viability in a dose-dependent manner. Enhanced cell death f ollowing Palbociclib treatment was also detected in a time-dependent fashion. It is supported by the decre ased IC_{50} (half maximal inhibitory concentration) over duration of treatment. Cell morphological changes were observed, as it is evident by the formation of cytoplasmic vacuoles (Fig. 2, arrowheads indicate cyto plasmic vacuoles), which is a sign of cells undergoing autophagy.



Cell line/time	24 hr	48 hr	72 hr
A549	27.04 μM	14.52 μM	6.60 μM
H358	25.04 μM	13.05 μM	8.51 μM

Figure 1. Palbociclib leads to increased cell death in NSCLC cell lines. A549 and H358 cells (5000 cells/ well) were exposed to the indicated doses (0, 5, 10, 15, 25, 35 μM) of Palbociclib for 24, 48 and 72 hou rs. Cell viability was evaluated by MTT assay. SF, serum free. IC_{50} was calculated by Prism 6 software. V alues are shown as the mean ± SD (n = 6).

Cell viability (% of control) = (treatment group $OD_{570\ nm}$ / control group $OD_{570\ nm}$) x 100

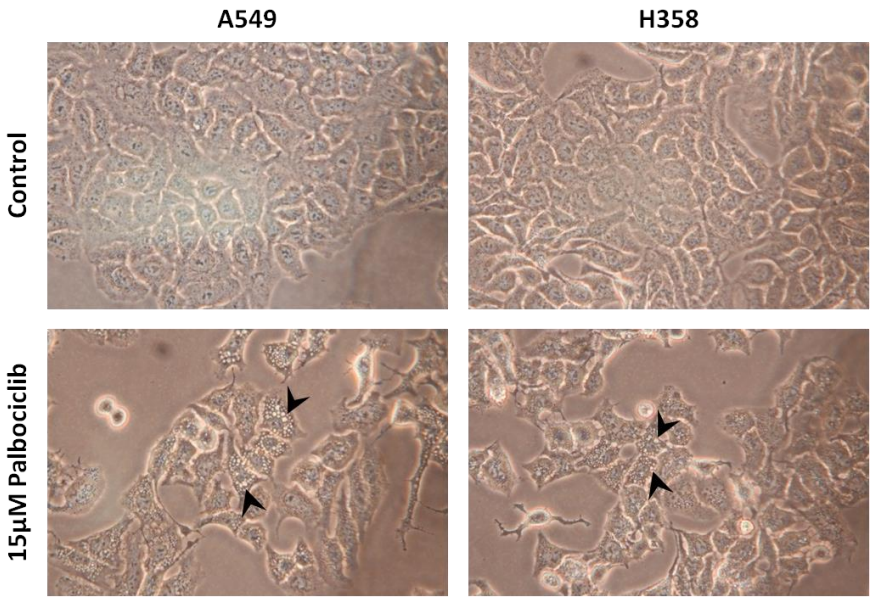


Figure 2. Palbociclib treatment results in morphology changes of NSCLC cells. A549 and H358 cells (5x 10^5 cells) were incubated with DMSO (0.3%) or 15 μM of Palbociclib for 24 hours. Images of cell morphology were recorded (400X magnification). Arrowheads indicate cytoplasmic vacuoles.

Secondly, we wanted to see whether or not Palbociclib is capable of inhibiting the clonogenic potential of NSCLC cells. In my experiment, it has caught our attention that Palbociclib is able to inhibit the clonogenic potential of NSCLC cells. Anchorage-independent growth describes a common feature of cancer cells to proliferate independent of a solid substrate. In order to investigate the effects of Palbociclib on the clonogenic potential of NSCLC cells, we performed the colony formation assays. As indicated in Fig.3, Palbociclib treatment inhibited colony formation of A549 and H358 lung cancer cells. The results implied the potential of Palbociclib to inhibit malignant transformation in NSCLC cells.

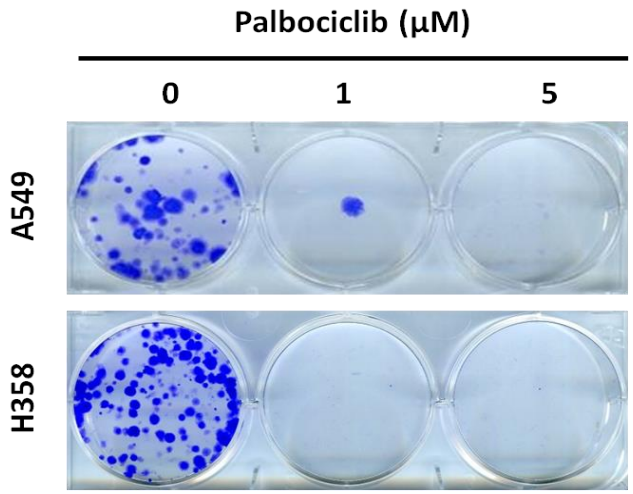


Figure 3. Palbociclib reduces colony formation in NSCLC cells. A549 and H358 cells were seeded into 6-well plate (1000 cells/well) and exposed to different doses of Palbociclib for 14 days. Colonies were fixed and stained with crystal violet staining solution. Representative images of colony formation in each group are shown.

Next, we want to figure out whether or not Palbociclib is capable of inducing apoptosis and autophagy th at leads to cell death in NSCLC cells *in vitro*. We further investigated if Palbociclib could induce apoptos is, a common mechanism of programmed cell death triggered by anti-cancer drugs, in NSCLC cells. Cell death Enzyme-linked immunosorbent assay (ELISA) was utilized to assess cytoplasmic nucleosomes, whi ch were released from the nucleus during apoptotic cell death. As shown in Fig. 4, more cytoplasmic nucl eosomes were detected with increased concentration of Palbociclib. This suggested that Palbociclib could induce apoptosis in NSCLC cells. We then examined the correlation between Palbociclib and cell death i n NSCLC cells at a molecular level. The expression of cleaved caspase 8, caspase 9, caspase 3, and PAR P-1 were determined as an indicator of apoptosis. We found that the upregulation of cleaved caspases and PARP-1 were associated with increased Palbociclib concentration (Fig. 5) and treatment duration (Fig. 6). Enhanced cleavage of caspase 9, but not caspase 8, was seen from the experiment, suggesting that Palboc iclib triggered apoptotic cell death through the intrinsic apoptotic pathway. Enhanced LC3 conversion (fr om LC3-I to LC3-II) was also seen following Palbociclib treatment (Fig. 5 & Fig. 6), indicating that Palb ociclib may trigger autophagy, in addition to the initiation of apoptosis. The autophagy inhibitor 3-MA (F ig. 7A) and the apoptosis inhibitor z-VAD-FMK (Fig. 7B) were added to Palbociclib treated A549 cells. The additions of both inhibitors reduced the effect of palbociclib on the cell viability. These data suggest t hat Palbociclib reduced cell viability through the induction of apoptotic and autophagic cell death.

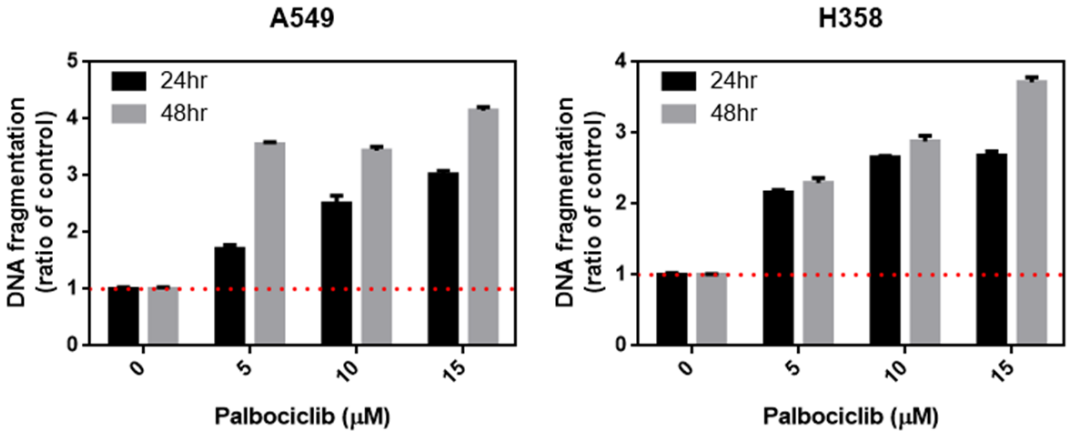


Figure 4. Palbociclib induces apoptotic cell death in NSCLC cells. A549 and H358 cells (5000~10000 ce lls/well) were treated with Palbociclib at the indicated concentrations (0, 5, 10, 15, 25 μM) for 24 or 48 hours. Apoptotic cell death was assessed by using Cell Death ELISA Kit. Columns, mean; bars, SD (n = 4).

DNA fragmentation (ratio of control) = (treatment group Delta OD – background Delta OD) / (control group Delta OD – background Delta OD)

Delta OD = $OD_{405\ nm}$ - $OD_{490\ nm}$; background value (incubation buffer)

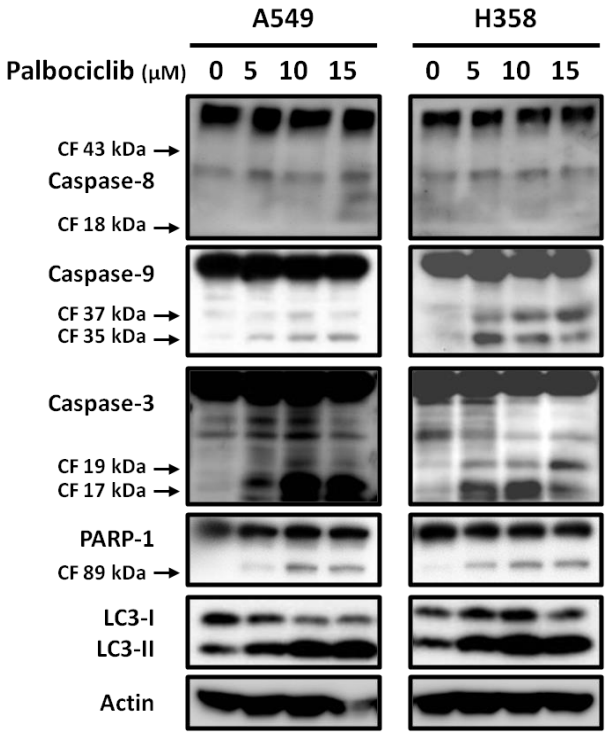


Figure 5. Palbociclib induces apoptosis and autophagy in NSCLC cells *in vitro*. A549 and H358 cells were seeded into 6-cm dish ($5x10^5$ cells) and treated with the indicated concentrations of Palbociclib for 24 hours. Cell lysates were then subjected to western blot analyses. CF, cleaved form (activated form). β-actin was used as loading control.

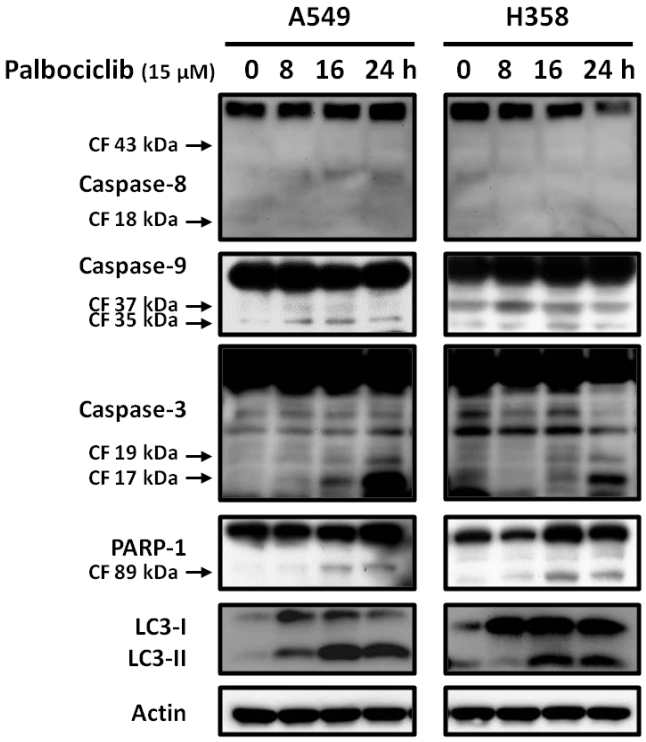


Figure 6. Palbociclib-triggered apoptosis and autophagy increases with treatment duration. A549 and H358 cells were seeded into 6-cm dish ($5x10^5$ cells) and treated with 15 μM Palbociclib for 0, 8, 16, 24 hours. Cell lysates were harvested for western blot analyses of apoptosis and autophagy markers. CF, cleaved form (activated form). β-actin was used as a loading control.

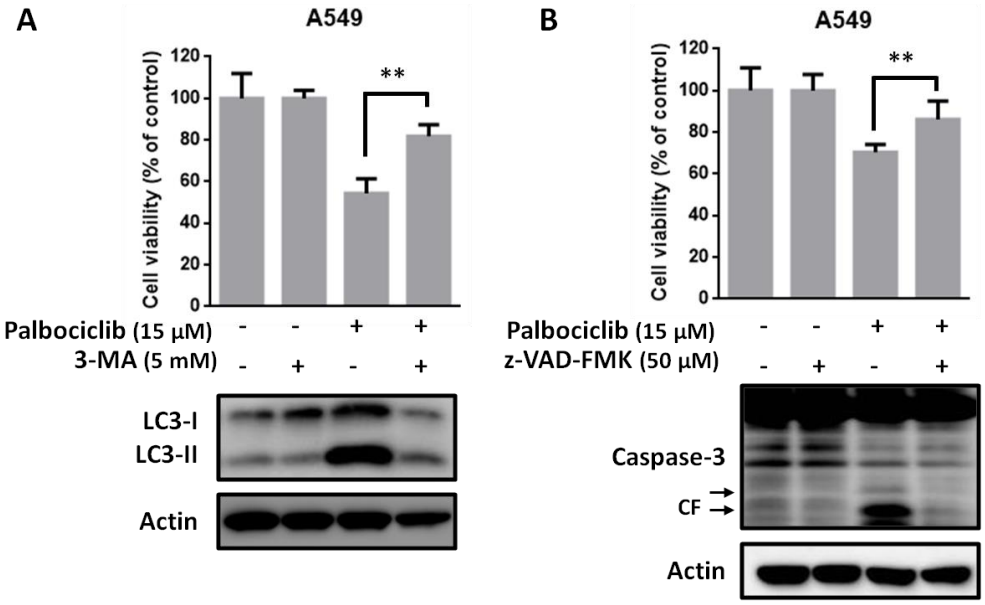


Figure 7. Palbociclib-induced apoptosis and autophagy contributes to cell death. (A) The autophagy inhibitor, 3-methyladenine (3-MA) rescued A549 cells from Palbociclib-induced autophagy. A549 cells were pretreated with 3-MA (5 mM) for 2 hours and treated with Palbociclib (15 μM) for another 24 hours. (B) Pan-caspase inhibitor, z-VAD-FMK reduced the apoptotic effects of Palbociclib. A549 cells were preincubated with z-VAD-FMK (50 μM) for 2 hours before Palbociclib treatment. Cells were then analyzed by MTT assay (upper panel) and western blot analyses (lower panel). Columns, mean; bars, SD (n = 8). Statistical significance was evaluated by Student's *t*-test. **, $p < 0.01$.

As described previously, both apoptotic and autophagic cell death play an important role in mediating Palbociclib's effects on lung cancer cells. We next examined if AMP-activated protein kinase (AMPK) was involved in the mode of actions of Palbociclib on lung cancer cells. The AMPK assumes a critical role in regulating cellular processes, including glucose uptake, lipid metabolism, apoptosis, and autophagy. The upregulation of p-AMPK (Thr172) following Palbociclib treatment implied that Palbociclib enhanced the activity of AMPK, in a dose-dependent (Fig. 8) and time-dependent (Fig. 9) manner. The phosphorylation of UNC-51 like kinase-1 (ULK1), a downstream molecule of AMPK participated in autophagy, was also elevated upon Palbociclib treatment (Fig. 8 & Fig. 9). We further silenced AMPK by exploiting an inhibitor of AMPK (Fig. 10A) and siRNA against AMPK (Fig. 10B). Inhibition of AMPK activity by compound c (Fig. 10A) and AMPK expression knockdown by siRNA (Fig. 10B) both relieved NSCLC cells from Palbociclib-induced apoptosis and autophagy. These data suggest that AMPK play a key role in mediating Palbociclib's effects on lung cancer cells.

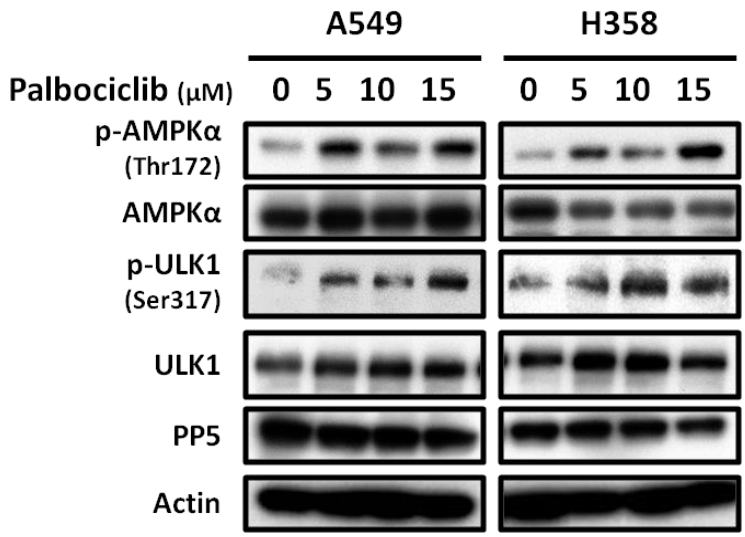


Figure 8. Palbociclib treatment enhances the phosphorylation of AMPK in NSCLC cells. A549 and H358 cells were treated with pre-determined concentrations of Palbociclib for 24 hours. Cell lysates were subje cted to western blot analyses. β-actin was used as the loading control.

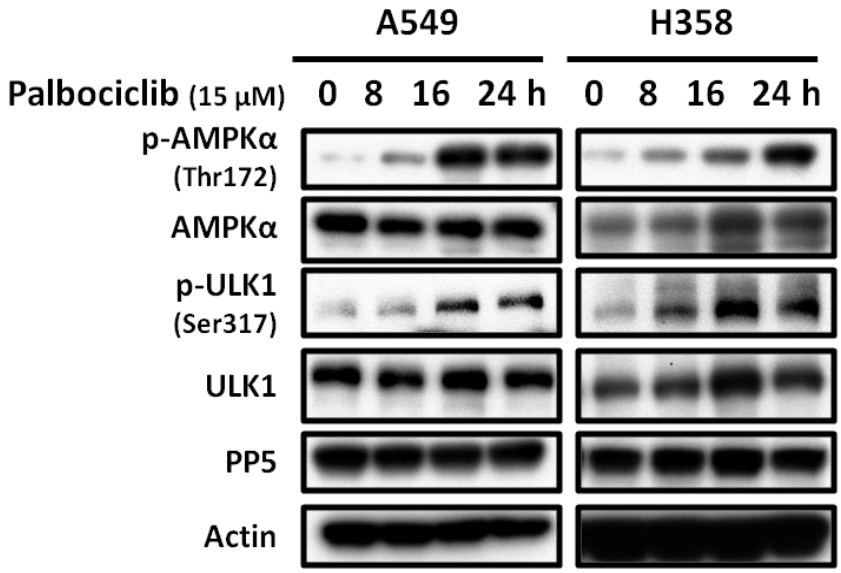


Figure 9. The phosphorylation of AMPK increases with duration of Palbociclib treatment. A549 and H35 8 cells were treated with 15 μM of Palbociclib for 0, 8, 16, 24 hours. Cell lysates from different time po ints were analyzed by western blotting.

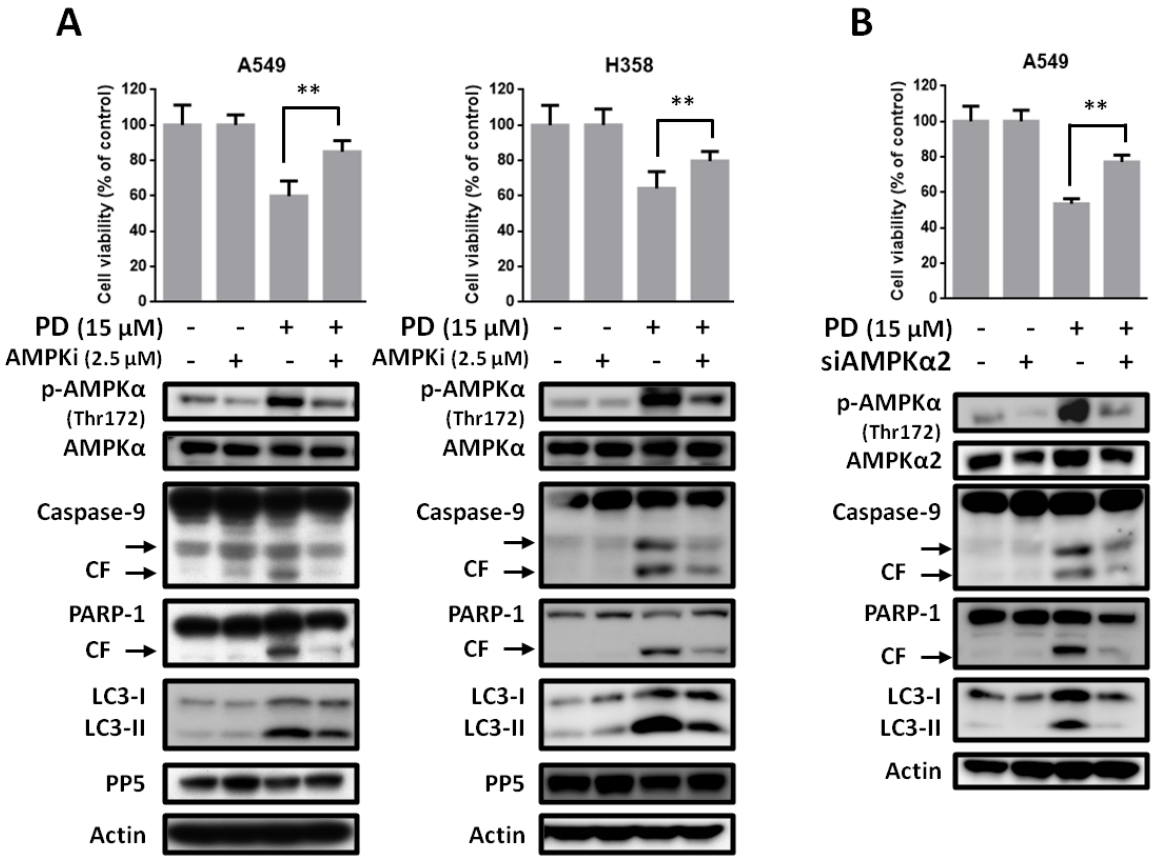


Figure 10. AMPK mediates Palbociclib-induced apoptotic cell death and autophagy. (A) The AMPK inhibitor (AMPKi), compound c, rescued NSCLC cells from Palbociclib-induced cell death. A549 and H358 cells were preincubated with AMPKi (2.5 μM) for 2 hours followed by 15 μM Palbociclib for 24 hours. (B) Silencing AMPK by siRNA counteracted Palbociclib-triggered cell death. A549 cells were transfected with control siRNA or AMPK siRNA before being treated with 15 μM Palbociclib for 24 hours. After that, cells were analyzed by MTT assay (upper panel) and western blotting (lower panel). Columns, mean; bars, SD (n = 8). Statistical significance was evaluated by Student's *t*-test. **, $p < 0.01$.

Furthermore, we wanted to find out how Palbociclib activates the phosphorylation of AMPK. In this report, we found out PP5 play a key role in mediating the effect of Palbociclib on AMPK activation. Palbociclib reduced PP5 activity, which resulted in the enhancing of phosphorylation of AMPK and cell death. Reduced activity of cellular protein phosphatase 5 (PP5), a phosphatase which negatively regulated the phosphorylation of AMPK, was observed in NSCLC cells incubated with Palbociclib (Fig. 11A). Diminished PP5 activity was also detected upon incubation of Palbociclib with PP5-containing A549 cell lysates (Fig. 11B). However, the expression of PP5 remained unchanged (Fig. 8 & Fig. 9) after Palbociclib treatment. In order to validate the role of PP5 in Palbociclib-induced AMPK phosphorylation and cell death, a PP5 agonist (Fig. 12A) arachidonic acid (AA) and ectopic expression of PP5 (Fig. 12B) were introduced. The a

ctivation of PP5, or PP5 overexpression, both reduced the phosphorylation of AMPK and rescued the N SCLC cells from Palbociclib-induced cell death. The data described above indicated that, Palbociclib trea tment reduced PP5 activity and contributed to increased phosphorylation of AMPK and cell death in NSC LC cells.

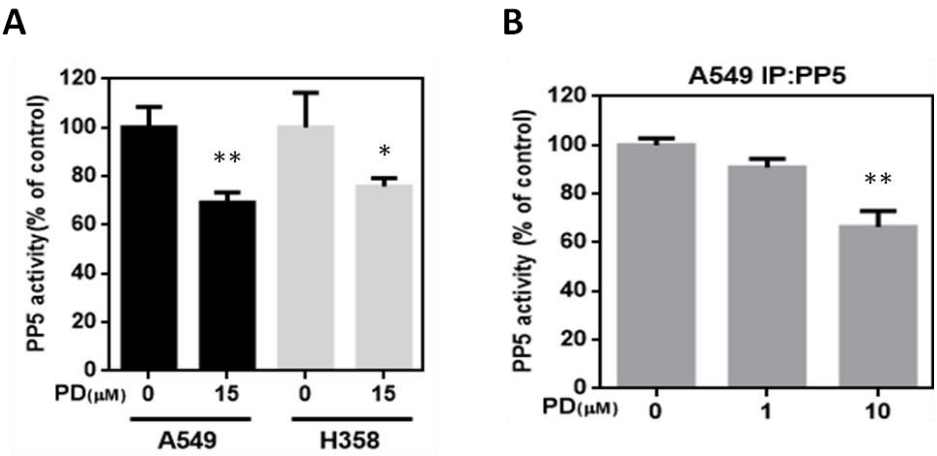


Figure 11. Palbociclib reduces the activity of PP5 . (A) Palbociclib inhibited cellular PP5 activity in A54 9 and H358 cells. Cells were treated with 15 μM Palbociclib for 24 hours and then cell lysates were im munoprecipitated with anti-PP5 antibody. The immunoprecipitants were subjected to a Serine/Threonine phosphatase assay. (B) Palbociclib suppressed PP5 activity in PP5-containing A549 lysates. A549 lysates were collected and immunoprecipitated with anti-PP5 antibody. The immunoprecipitants were then incub ated with 0, 1, 10 μM of Palbociclib and PP5 activity was measured. Data shown are mean ± SD (n = 3) by using Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$.

PP5 activity (% of control) = (treatment group fluorescence – background fluorescence) / (control group fluorescence - background fluorescence) x 100 ; background value (assay buffer)

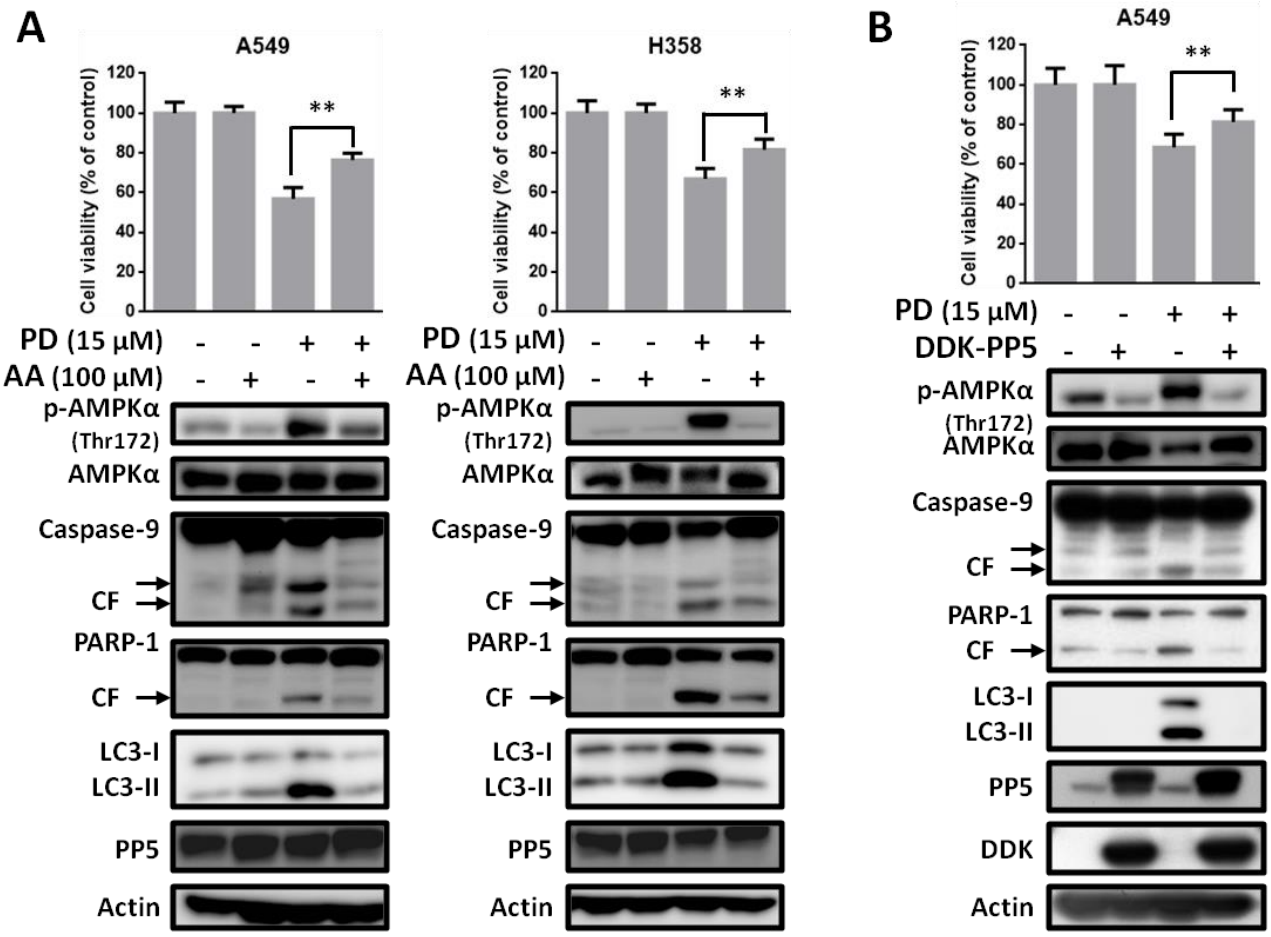


Figure 12. Palbociclib-reduced PP5 activity contributes to the enhanced expression of p-AMPK and cell death. (A) The PP5 activator, arachidonic acid (AA) reversed the effects of Palbociclib on p-AMPK and cell death. A549 and H358 cells were pretreated with AA (100 μM) for 2 hours followed by 15 μM Palbociclib treatment for 24 hours. (B) Ectopic expression of PP5 rescued NSCLC cells from Palbociclib induced AMPK phosphorylation and cell death. A549 cells were transfected with vector or DDK-PP5 for 24 hours and treated with 15 μM Palbociclib for another 24 hours. Next, cells were analyzed by MTT assay (upper panel) and western blot analyses (lower panel). Columns, mean; bars, SD (n = 8). Statistical significance was evaluated by Student's *t*-test. **, $p < 0.01$.

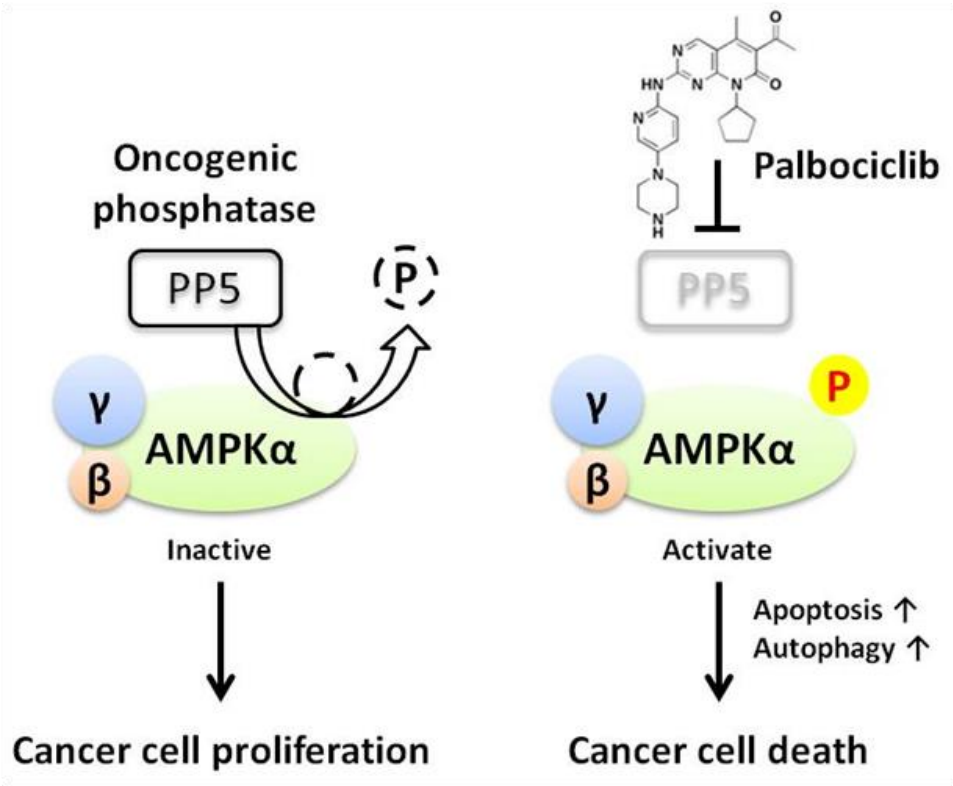


Figure 13. Schematic representation of the oncogenic role of PP5 and the anti-NSCLC mechanism of Palbociclib. PP5 promotes NSCLC proliferation through the dephosphorylation and inactivation of AMPK. By inhibiting PP5 activity, Palbociclib activates AMPK and triggers apoptosis, as well as autophagy, leading to the subsequent NSCLC cell death.

The data corroborates with results from our *in vitro* studies that Palbociclib treatment reduces the activity of PP5, leading to the phosphorylation of AMPK and NSCLC cell death (Fig. 13).

（二）討論

Palbociclib was the first in a class of CDK4/6 inhibitors, including Ribociclib and abemaciclib, to display clinical benefits and received approval to treat advanced breast cancer patients.. Regarding to NSCLC, a report demonstrated that Palbociclib enhanced the anti-cancer activity of selumetinib, a MEK inhibitor which repressed the phosphorylation of ERK, in RAS-driven NSCLC [4]. Prolonged Palbociclib treatment resulted in NSCLC cell growth inhibition [5]. Palbociclib plus Trametinib, a MEK inhibitor, sensitized KRAS-mutant NSCLC cells to radiotherapy [6]. Of note, the regime of Palbociclib in combination with MEK inhibitors were currently tested clinically in patients with KRAS mutant NSCLC (NCT02022982 and NCT03170206) [7].

We assessed the effects of Palbociclib, as a single agent, against NSCLC cells. We found that Palbociclib exhibited modest anti-NSCLC activities, as evidenced by reduced cell viability (Fig. 1). Palbociclib-induced NSCLC cell death, was at least partially mediated by apoptosis and autophagy, since the autophagy inhibitor 3-MA and the apoptosis inhibitor z-VAD-FMK rescued the cell death triggered by Palbociclib treatment (Fig. 7). Interestingly, the anti-NSCLC activities displayed by Palbociclib may beyond simply suppress CDK4/6. By incubating the NSCLC cells with Palbociclib, or incubating the PP5-containing NSCLC cell lysates with Palbociclib, we found that Palbociclib treatment led to reduced PP5 activity (Fig. 11). PP5 is a negative regulator of the phosphorylation of AMPK, whose activation may oppose tumor growth [8]. Indeed, we found that Palbociclib treatment upregulated the expression of p-AMPK (Thr172), in a dose- (Fig. 8) and time course-dependent fashion (Fig. 9). Application of a PP5 agonist arachidonic acid (AA), or ectopic expression of PP5, both downregulated p-AMPK and rescued NSCLC cells from Palbociclib-triggered cell death (Fig. 12), highlighted the role of PP5 in mediating Palbociclib-elicited signaling cascades. Compound c incubation to inhibit AMPK and AMPK knockdown, both relieved NSCLC cells from Palbociclib-induced autophagy and apoptosis (Fig. 10), further validated the role of AMPK in Palbociclib-induced NSCLC cell death.

PP5 is a serine/threonine kinase, and also a member of the phosphoprotein phosphatases (PPP) family. PP5 is involved in stress-, as well as hormone-response, and malignant cell growth [9]. Clinical samples showed that the expression of PP5 is significantly higher in the NSCLC tumor regions, compared to the normal tissues [10]. Overexpression of PP5 may provide survival benefits to NSCLC cells [11]. On the other hand, high levels of p-AMPK in NSCLC patients were correlated to the increased survival [12]. Hence, p-AMPK may function as a biomarker, for further Palbociclib trials in NSCLC patients. Besides, the phosphorylation and activation of AMPK may sensitize cancer cells to radiotherapy [13], and it has been indeed mentioned that Palbociclib may sensitize NSCLC cells to radiotherapy [6].

We herein reported that Palbociclib treatment triggered NSCLC cell death, through the inhibition of PP5 activity and upregulation of p-AMPK. Our studies on Palbociclib may lay a foundation for the development of Palbociclib against NSCLC, as a single agent, or in combination with other treatment modalities.

四、結論與應用

In this study, we investigated a new drug called Palbociclib, a CDK 4/6 inhibitor that has recently been a pproved for patients with advanced breast cancer. As Palbociclib showed anti-cancer activity in several tu mor models besides breast cancer, we examined its activity against Non-Small-Cell-Lung-Cancer (NSCL C) cells and explored the underlying mechanism.

We found that Palbociclib exhibited anti-NSCLC activities, such as reduced cell viability following Palbo ciclib treatment in vitro. Palbociclib-induced NSCLC cell death was mediated by apoptosis and autophag y, which were both triggered by the activation of p-AMPK. Palbociclib activated p-AMPK through the ef fect on suppressing the activity of PP5.

From this study, we have shown that Palbociclib exhibited anti-cancer effects on NSCLC cells and identif ied a novel mechanism by which Palbociclib induced cell death by modulating the PP5-AMPK signaling pathway. Our new findings on may provide a mechanistic foundation for developing Palbociclib as a sing le agent or as in combination with agents for the treatment of NSCLC. Hopefully, our research can be ben eficial to studies of NSCLC.

五、參考文獻

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【評語】090019

Palbociclib 是剛核准的乳癌藥物，主要是藉由抑制 CDK4/6 來延緩乳癌生長。我們發現 Palbociclib 能有效地抑制肺癌細胞株的生長和死亡。特別的是，Palbociclib 不僅誘發肺癌細胞凋亡(apoptosis)，亦誘發肺癌細胞自噬死亡(Autophagy)。因為 AMPK 能同時影響細胞凋亡與自噬死亡，進一步研究 AMPK 在 Palbociclib 誘發癌細胞死亡中的角色，發現 Palbociclib 主要是藉由抑制去磷酸酶 PP5 的活性，來增加 AMPK 的磷酸化，進而活化 AMPK，誘發肺癌細胞產生凋亡與自噬。

建議進一步了解與此研究的異同：Mol Oncol. 2017 Aug;11(8):1035. Palbociclib induces activation of AMPK and inhibits hepatocellular carcinoma in a CDK4/6-independent manner.