RESEARCH PAPER

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A CDK4/6 inhibitor enhances cytotoxicity of paclitaxel in lung adenocarcinoma cells harboring mutant KRAS as well as wild-type KRAS

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Keywords: NSCLC, KRAS, CDK4 siRNA, CDK4/6 inhibitor, paclitaxel, combination, synergistic

Abbreviations: CDK, cyclin-dependent kinase; siRNA, short interfering RNA; Rb, retinoblastoma; NSCLC, non-small cell lung cancer; EGFR TKI, growth factor receptor tyrosine kinase inhibitor; CI, combination index; RNAi, RNA interference

The KRAS gain-of-function mutation confers intrinsic resistance to targeted anticancer drugs and cytotoxic chemotherapeutic agents, ultimately leading to treatment failure. KRAS mutation frequency in lung adenocarcinoma is ~15–30%. Novel therapeutic strategies should be developed to improve clinical outcomes in these cases. Deregulation of the p16/cyclin-dependent kinase (CDK) 4/retinoblastoma (Rb) pathway is frequently observed in various cancers and it represents an attractive therapeutic target. We compared the antitumor efficacy of genetically knocked-down CDK4 and a pharmacological inhibitor of CDK4/6, CINK4, in KRAS mutation-positive lung adenocarcinoma cells. We also investigated changes in anti-proliferative activity and downstream molecules with these treatments in combination with paclitaxel. CDK4 short interfering RNA (siRNA) significantly increased paclitaxel sensitivity in KRAS mutation-positive H23 cells. CINK4 demonstrated concentration- and time-dependent anti-proliferative activity in 5 adenocarcinoma lines. CINK4 induced G_1 arrest by downregulating the p16/cyclin D1/Rb pathway, resulting in apoptotic induction via increased expression of cleaved caspase3, cleaved PARP and Bax. Combined CINK4 and paclitaxel produced synergistic anti-proliferative activity and increased apoptosis through reduced cyclin D1 and Bcl-2 in KRAS mutation-positive cancer cells. These data suggest CDK4 is a promising target for development of anticancer drugs and CINK4 combined with paclitaxel may be an effective therapeutic strategy for enhancing anti-tumor efficacy in KRAS mutation-positive lung adenocarcinoma.

Introduction

Mutations in the KRAS oncogene have been implicated in the development of various malignancies including non-small cell lung cancer (NSCLC).[1] Approximately 15–30% of lung adenocarcinoma patients possess a gain-of-function mutation in KRAS codons 12, 13, or 61;[2,3] the majority of KRAS mutations occur at codon 12.[4] Many clinical researchers have reported that KRAS mutation positivity confers poor prognosis with intrinsic resistance to cytotoxic chemotherapeutic drugs.[5] Targeted anticancer drugs including epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) exhibit antitumor efficacy, causing a paradigm shift in the treatment of advanced NSCLCs. However, they are only effective in the presence of activating EGFR mutations, not KRAS mutations.[6] In addition, chemotherapeutic outcomes for NSCLC patients harboring mutationally activated KRAS are also disappointing.[6-8] Currently, MEK1/2 inhibitor or sorafenib alone and in combination with cytotoxic drugs have shown promise in treatment of KRAS mutation-positive lung tumors models[9,10] but these treatment schedules have not been adopted in clinical practice. An effective therapeutic strategy for KRAS mutation-positive lung adenocarcinoma is urgently needed to improve clinical outcomes.

KRAS mutations constitutively activate the RAS/RAF/ERK signaling pathway.[11] Activation of ERK1/2 modulates the activity of target transcription factors[12] such as cyclin D1 and the cyclin D1-CDK4 complex.[13,14]

The p16/cyclin D1/CDK4/Rb signaling pathway is frequently altered in NSCLCs. The CDKN2A/p16 gene is a candidate tumor suppressor in wild-type Rb malignancies; p16 binds CDK4 and inhibits catalytic activity of the CDK4/cyclin D complex.[15] Rb and $p16^{INK4A}$ expression in lung cancer are inversely correlated; frequently, Rb-positive NSCLCs have little or no detectable $p16^{INK4A}$ protein due to homozygous deletions, mutations and methylation of CpG islands.[16,17] Loss of $p16^{INK4A}$ frees these CDKs from inhibition, permitting constitutive phosphorylation of Rb and inactivation of its cell growth inhibitory properties. In

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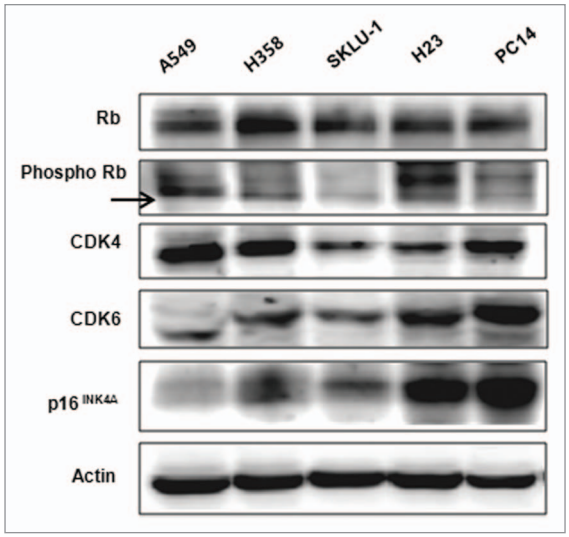

Figure 1. Baseline expression of p16/CDK4/Rb signaling molecules in 5 cancer cell lines. Constitutive basal expression of Rb, p-Rb, CDK4, CDK6, and $p16^{INK4A}$ were detected by western blotting. Actin was used as a loading control.

addition, overexpression of p16 blocks tumor cells in S phase and inhibits RAS-induced cell proliferation.[18,19]

The activity of CDK4, negatively regulated by $P16^{INK4A}$, is required for tumor progression in a $KRAS^{G12V}$ induced lung adenocarcinoma model. RAS and CDK4 co-expression promotes Rb phosphorylation, ultimately resulting in human invasive neoplasm.[20] Ablation of CDK4 selectively decreases the number of $KRAS^{G12V}$-expressing cells.[21] Therefore, CDK4 may be an attractive drug candidate for KRAS mutation-positive NSCLC.

PD0332991, a CDK4/CDK6 selective inhibitor with significant therapeutic activity in a $KRAS^{G12V}$-induced NSCLC model[22] and is being developed with other chemotherapeutic agents in clinical trials.[22-24]

Paclitaxel has potent cytotoxic activity as it stabilizes the microtubule cytoskeleton, thus blocking cell cycle progression. Because paclitaxel alone has a 21–24% response rate in advanced NSCLCs,[25,26] it is commonly used in combination with other anticancer agents.

In this study, we investigated whether selective molecular (siRNA) or pharmacological inhibition (CINK4, a small molecule targeting CDK4 and CDK6) of CDK4 activity may enhance the antitumor activity of paclitaxel in KRAS mutation-positive lung adenocarcinoma cell lines.

Results

Baseline expression of p16/CDK4/Rb signaling molecules. We observed different baseline expression levels for Rb, phosphorylated Rb (p-Rb), CDK4, CDK6 and $p16^{INK4}$ proteins in 5 cell lines (**Fig. 1**). Rb and p-Rb were present in all cell lines but p-Rb expression was lowest in SKLU-1 cells. The CDK4 is highest in A549 and H358 cells, while $p16^{INK4}$ is highest in H23 and PC14 cells. Similarly, CDK4 and CDK6 expression do not correlate.

siRNA knockdown of CDK4 and cell cycle distribution. To understand the molecular function of CDK4 in KRAS mutation-positive lung cancer cells, we used siRNA to knockdown CDK4 in H23 cells. We confirmed that CDK4 siRNA efficiently abolished p-Rb as well as CDK4 mRNA and protein expression (**Fig. 2A and B**). We also analyzed the cell cycle distribution in cells transfected with CDK4 siRNA. The cells in G_0/G_1 phases increased ($P < 0.05$) and the cell population in S and G_2/M phase decreased (**Fig. 2C**).

CDK4 knockdown enhances sensitivity to paclitaxel. When exposed to various concentrations of paclitaxel (0–10 nM), the inhibitory effect on cell proliferation was significantly higher in CDK4 siRNA-transfected H23 cells than in control siRNA-transfected H23 cells. Thus, selective inhibition of CDK4 may increase paclitaxel sensitivity in KRAS mutation-bearing H23 cells ($P < 0.05$ for paclitaxel 1 and 10 nM; $P < 0.01$ for paclitaxel 3 and 5 nM) (**Fig. 3**).

Anti-proliferative efficacy of CDK4/6 inhibitor CINK4. We measured the antitumor efficacy of CINK4 to compare genetic silencing vs. pharmacologic inhibition of CDK4 in 4 KRAS mutant and 1 KRAS wild-type cell line. CINK4 treatment at various concentrations (~0.1–40 μM) yielded dose- and time-dependent cytotoxicity in all 5 cell lines, regardless of the presence or absence of KRAS mutation. The IC_{50} values of CINK4 at

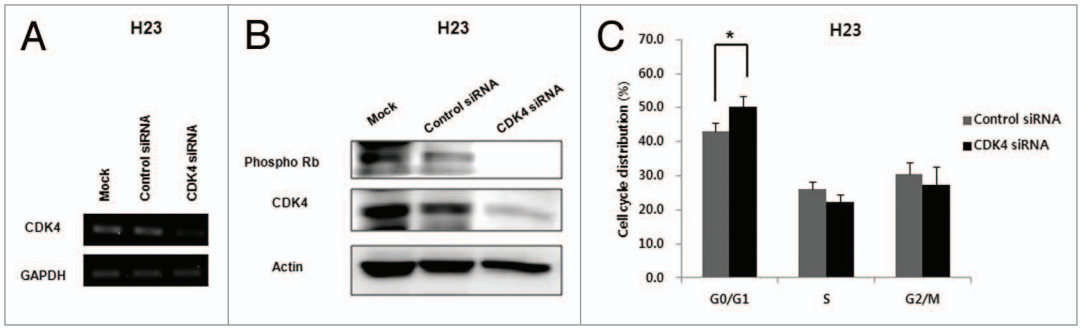

Figure 2. Efficient inhibition of CDK4 mRNA and protein and changes in cell cycle distribution in H23 cells transfected with CDK4 siRNA. (**A**) Expression of CDK4 mRNA examined by RT-PCR 48 h after CDK4 siRNA transfection. GAPDH was used as a loading control. (**B**) Expression of CDK4 and pRb protein was significantly reduced 48 h after CDK4 siRNA transfection. Actin was used as a loading control. (**C**) Cell cycle distribution. G_0/G_1 phase increased 48 h after CDK4 siRNA transfection of H23 cells. Each experiment was performed in triplicate. Data represent the mean ± SD. Statistically significant differences between the CDK4 siRNA and control are presented as *($P < 0.05$).

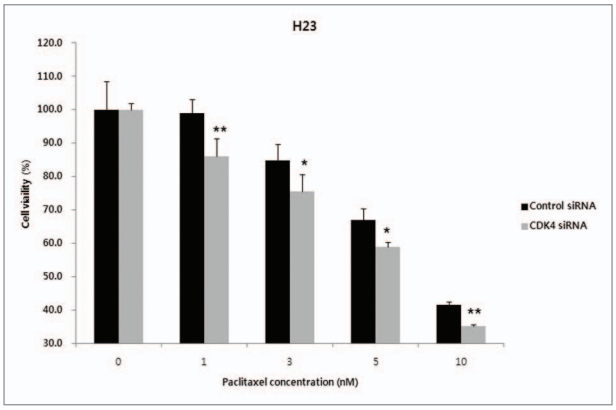

Figure 3. Enhanced anti-proliferative effect of paclitaxel in CDK4 siRNA-transfected H23 cells. When exposed to the different concentrations of paclitaxel (1, 3, 5, and 10 nM), a concentration-dependent increase of anti-proliferative effects was observed in the CDK4 siRNA-transfected cells. Each experiment was performed in triplicate. Data represent the mean ± SD. Statistically significant differences between the CDK4 siRNA and control are presented as * ($P < 0.05$) and ** ($P < 0.01$).

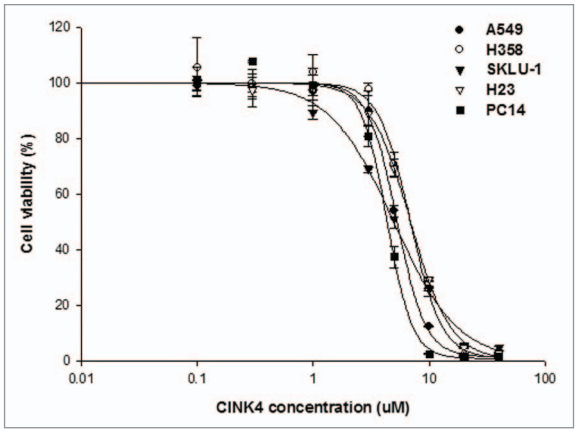

Figure 4. Anti-proliferative effect of a CDK4/6 inhibitor, CINK4, in 5 lung adenocarcinoma cell lines. When treated with various concentrations of CINK4 (0.1–40 μM), the anti-proliferative effects of CINK4 were similar for each cell line at 72 h. Cell viability was quantitatively measured by SRB assay and presented as a percentage of the control cell population. Each experiment was performed in triplicate. Data represent the mean ± SD.

72 h were 4–7 μM. Cell proliferation in all tumor cells was completely inhibited by > 20 μM CINK4 (**Fig. 4**). The IC_{50} values of CINK4 at 24, 48, and 72 h are summarized in **Table 1**.

CINK4 induces G_0-G_1 arrest through downregulation of phosphorylated Rb and induced apoptosis. We analyzed cell cycle distributions following 48 h CINK4 treatment in 5 lung cancer cell lines. In A549 and H23 cells, the cell population in G_0/G_1 phase increased but S and G_2/M phase decreased in a concentration-dependent manner (**Fig. 5A**). A similar cell cycle distribution pattern was observed in the other 3 cell lines (data not shown). In addition, the subG1 fraction of H23 cells significantly increased at 10 μM CINK4 (15.8 ± 3.8% vs. 4.6 ± 0.6% for control, $P < 0.01$); this was not observed in A549 cells (**Fig. 5A**). The subG1 fraction of H358 and PC14 cells (but not SKLU-1) significantly increased (data not shown). Double staining with annexin V-FITC and PI was used to determine whether the increased subG1 fraction was due to induction of apoptosis. In H23 but not A549 cells, apoptotic induction occurred at 10 μM CINK4 for 72 h (27.2 ± 3.1% vs. 9.9 ± 0.5% for control, $P < 0.001$) (**Fig. 5B**). CINK4 also induced apoptosis at same concentration in H358 and PC14 cells, but not in SKLU-1 cells (data not shown).

Prolonged 72 h exposure of A549 and H23 cells to CINK4 reduced phosphorylated Rb and cyclin D1 in a concentration-dependent manner. We obtained similar results in H358, SKLU-1 and PC14 cells (data not shown). Total Rb was reduced at higher concentrations (10 μM) in A549 and H23 cells. CINK4 had no inhibitory effect on expression of phosphorylated ERK, nor did CDK4 and CDK6 have an effect on A549 and H23 cells (**Fig. 5C**). The remarkable increase in cleaved PARP and caspase 3 occurred at 10 μM CINK4 in H23 cells but not A549 cells. Bax expression was strongly increased but Bcl-2 expression was not significantly altered at 10 μM CINK4 in H23 and A549 (**Fig. 5C**).

CINK4 enhances paclitaxel sensitivity in KRAS mutation-bearing lung cancer cells. Paclitaxel (0.1–300 nM; 72 h) induced dose-dependent cytotoxicity in 3 KRAS mutation-positive lung cancer cell lines. The IC_{50} value was 3 nM in A549 and H23 cells and 40 nM in SKLU-1 cells (**Fig. 6A**). Even at 300 nM paclitaxel, 8.9 ± 1.6% and 9.0 ± 0.4% of A549 and H23 cells and 45.4 ± 3.2% of SKLU-1 cells remained alive.

Table 1. Sensitivity of NSCLC cell lines to CINK4

Time	A549	H358	SKLU-1	H23	PC14
24 h	10.2 ± 1.8	15.0 ± 1.7	21.8 ± 3.5	14.1 ± 0.2	8.0 ± 0.8
48 h	10.5 ± 0.2	10.1 ± 1.9	8.6 ± 0.9	9.0 ± 0.5	6.6 ± 0.8
72 h	6.1 ± 1.2	6.6 ± 0.5	5.0 ± 0.3	6.8 ± 0.4	4.3 ± 0.6

The IC_{50} values (μM) of each cell line, determined by SRB assays. Each experiment was performed in triplicate.

KRAS mutation-positive tumor cell growth inhibition was enhanced by a simultaneously combination of paclitaxel and CINK4, with a few exceptions (**Fig. 6B**). CINK4 (1, 3, 5, and 10 μM) combined with paclitaxel (1, 3, 5 and 10 nM) yielded CI values of 0.85 ± 0.23 for A549, 0.69 ± 0.19 for SKLU-1 and 0.98 ± 0.20 for H23. We observed synergistic anti-tumor activities with 10 μM CINK4 plus paclitaxel (1, 3, 5 and 10 nM). The CI values were 0.69 ± 0.10 for A549, 0.42 ± 0.10 for SKLU-1, and 0.87 ± 0.16 for H23 cells.

Paclitaxel combined with CINK4 increases apoptosis. A549 and H23 cells treated with paclitaxel, CINK4 and paclitaxel+CINK4, the proportion of apoptotic cells was 19.4 ± 0.2% ($P < 0.001$ vs. control), 10.2 ± 0.6% ($P < 0.01$ vs. control) and 45.0 ± 0.1% ($P < 0.001$ vs. paclitaxel single) in A549 cells and 26.1 ± 7.2% ($P < 0.01$ vs. control), 30.0 ± 1.3% ($P < 0.01$ vs. control) and 50.3 ± 0.8% ($P < 0.01$ vs. paclitaxel single) in H23 cells (**Fig. 7A**). Cleaved PARP, a marker of apoptosis, was increased in the combination vs. individual treatments. Expression of Bcl-2 and cyclin D1 decreased in the combination vs. individual treatments in A549 and H23 cells (**Fig. 7B**).

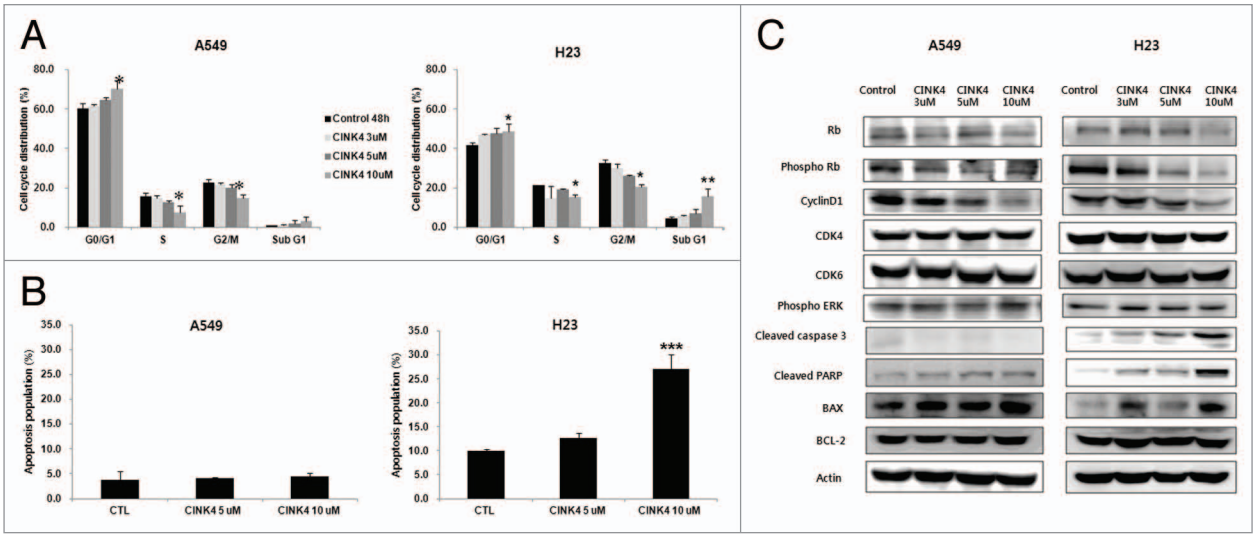

Figure 5. The effects of CINK4 on cell cycle distribution and apoptosis in KRAS mutation-bearing A549 and H23 cells. (**A**) Changes in cell cycle distribution were measured by flow cytometry after CINK4 treatment for 48 h. Each experiment was performed in triplicate. Data represent the mean ± SD. Statistically significant differences between CINK4 treatment groups and the control were presented as $*P < 0.05$ and $**P < 0.01$. (**B**) Apoptotic cells induced by CINK treatment (5 and 10 μM) were measured by flow cytometry. Annexin V-FITC/PI double staining was performed and Annexin V-positive apoptotic cells were counted by flow cytometry. Each experiment was performed in triplicate. Data represent the mean ± SD. Statistically significant differences between CINK4 treatment groups and controls are presented as $**P < 0.01$ and $***P < 0.001$. (**C**) The effects of CINK4 on cell cycle regulators and apoptosis-related molecules were evaluated by western blotting. A549 and H23 cells were treated with CINK4 (3, 5, and 10 μM) for 72 h. Actin was used as a loading control.

Discussion

Alternative signaling pathways may offer new therapeutic opportunities for treatment of KRAS mutation-positive lung adenocarcinoma. Selective inactivation of the p16/cyclin D1/CDK4/Rb signaling pathway is an important strategy for anticancer drug development. In this study, we investigated the anti-tumor effects of CDK4 suppression by molecular or pharmacologic inhibition in KRAS mutant cells. We also examined the impact of CINK4, a CDK4/6 inhibitor, in combination with paclitaxel.

RNA interference (RNAi) technology is a powerful approach to silence mammalian gene expression for studies of gene function; it also has therapeutic potential. In our study, transient downregulation of CDK4 with siRNA enhanced paclitaxel-induced cytotoxicity in KRAS mutation-bearing H23 cells; cytotoxic activity was statistically significant, however, potent cytotoxicity as we expected did not occur. CDK4 siRNA combined with paclitaxel may have been insufficient to induce potent anti-proliferative activity for several possible reasons. (1) Knockdown of CDK4 with transient CDK4 siRNA may not produce complete functional inhibition of CDK4. Various siRNAs targeting different sequences of the same gene can generate enormously variable inhibitory effects.[27] (2) Silencing of CDK4 may not be enough to enhance paclitaxel cytotoxicity because CDK6 can compensate for functional loss of CDK4. CDK4 and CDK6 are both essential for initiation of the cell cycle in response to various mitogenic stimuli. CDK4 is dispensable for proliferation in most cells, wherein CDK6 has a compensatory role.[28,29] In addition, ablation of CDK4 and CDK6 did not affect the expression of D-type cyclins.[29] As cyclin D1/CDK6 also play important roles in cell cycle progression in KRAS mutant cells with functional CDK4 loss, this may explain the modest cytotoxic effect of paclitaxel in our study. We used CINK4, a dual pharmacologic inhibitor of CDK4 and CDK6 in the next experiment.

We measured the growth inhibitory activity of CINK4 in 5 lung adenocarcinoma cell lines. Growth inhibition by CINK4 (IC_{50}) was similar in all cell lines, regardless of KRAS and p16 status. Thus, neither KRAS nor p16 status is directly correlated with sensitivity to CINK4. However, the cancer cell lines in our experiment all expressed active Rb. Our observation was consistent with previous reports that CINK4 had potent inhibitory activity on cell proliferation in the presence of Rb.[30]

The cell cycle is a process that tightly controls cell growth and proliferation. Disruption of the cell cycle process causes an imbalance between cell proliferation and cell death, thereby resulting in cancer development.[31] Our study demonstrated that CINK4 efficiently reduced phosphorylated Rb with the CDK4-specific Ser807 and Ser811 residues and cyclin D1 in a dose-dependent manner, subsequently leading to the accumulation of tumor cells in G_0/G_1 phase. However, neither CDK4 nor CDK6 expression changed in A549 and H23 cells treated with CINK4. Previously, Rajeev et al. reported that CINK4 did not reduce total expression of CDK4, but acts on CDK4 via phosphorylation of its tyrosine residue.[30]

An extensive list of signaling pathways regulate cyclin D1 and its transcription promoter binding sites.[32] ERK, a major downstream effector of RAS, is strongly associated with cell cycle entry particularly for the induction of cyclin D1 expression via downregulation of p27kip1.[33,34] Conversely, cyclin D1

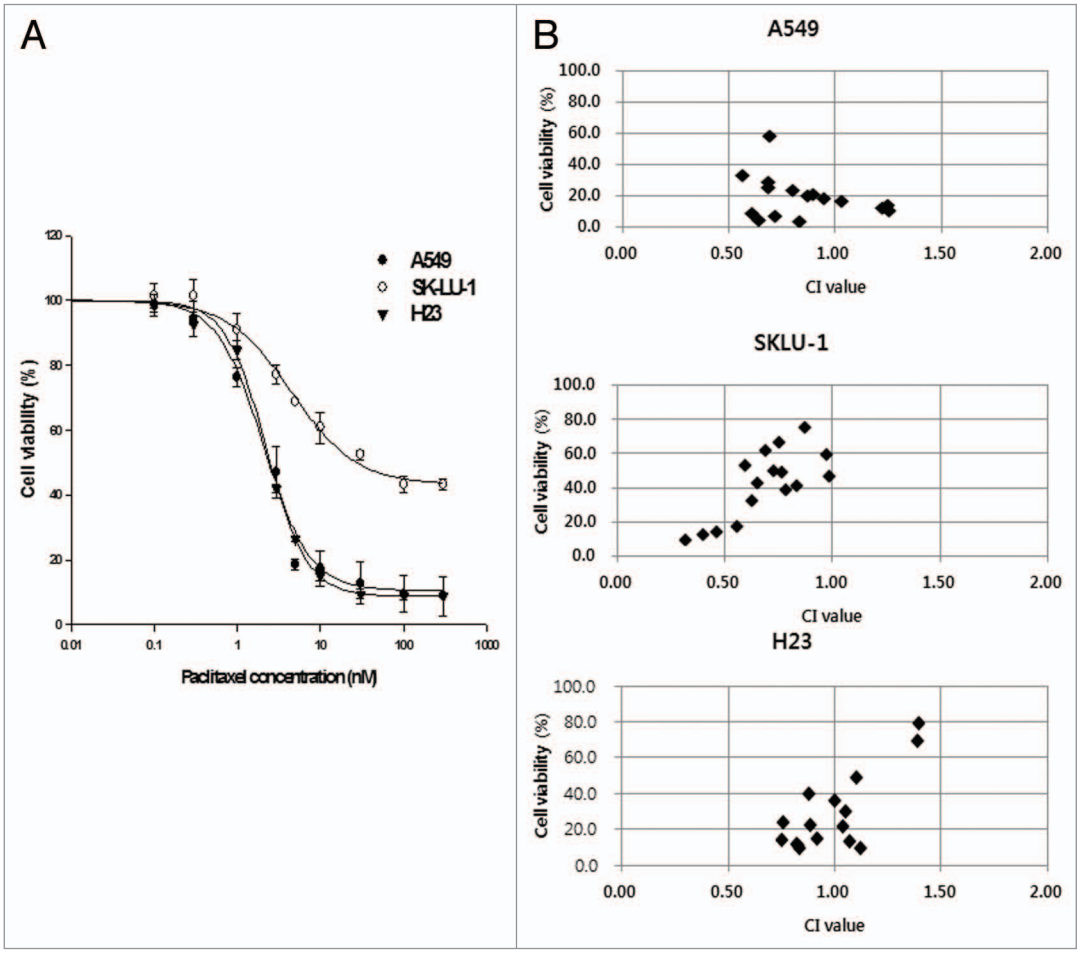

Figure 6. Anti-proliferative effects of paclitaxel alone and combined with CINK4 in 3 KRAS mutation-positive NSCLC cell lines. (**A**) Anti-proliferative effect of paclitaxel (0.1–300 nM) in 3 KRAS mutation-positive NSCLC cell lines at 72 h. (**B**) Paclitaxel (1, 3, 5 and 10 nM) was combined with CINK4 (1, 3, 5, and 10 μM) for 72 h in individual cell line. Interactions between paclitaxel and CINK4 were analyzed by combination index (CI). The calculated CIs were divided into 3 categories: CI < 1, CI = 1, or CI > 1, indicating synergistic, additive and antagonistic effects, respectively.

activity is independent of ERK expression.[34] In our experiment, CINK4 reduced cyclin D1, but did not affect its upstream regulator, phosphorylated ERK. These results suggest that the relationship between cyclinD1 and ERK is not a simple unidirectional linear one, at least in lung adenocarcinoma with KRAS mutations.

Key molecules in the cell cycle could serve as targets for anticancer agents to halt uncontrolled proliferation of tumor cells and to initiate apoptosis.[35] Our study demonstrated that apoptotic induction with increased Bax, cleaved PARP, and cleaved caspase3 occurred after CINK4 treatment (72 h, 10 μM) in H23, H358, and PC14, but not in A549 and SKLU-1. However, we found significantly increased cleaved PARP in A549 cells treated with CINK4 (72 h, 20 μM) (data not shown). CINK4 inhibits CDK4 in similar fashion of p16, an endogenous inhibitor of CDK4.[30] In present study, when treated with same concentration of CINK4, tumor cells had dissimilar apoptotic induction by CINK4. A previous study reported that $p16^{INK4}$ mediated apoptosis was induced when p16-deficient lung cancer cells was infected with an adenovirus vector (Adv/p16).[36] Our result suggested that apoptosis be positively correlated with expression level of endogenous $p16^{INK4}$. And our results are consistent with a previous report that low-concentration CINK4 also induced senescence, which was "bypassed" when cells were treated with either a higher dose or prolonged exposure to CINK4, both of which caused apoptotic induction.[30] Katsuda et al. reported that Rb was cleaved after activation of caspase-3; when Rb expression was significantly diminished, apoptosis began.[36] Our experiment showed that higher doses of CINK4 remarkably reduced Rb in A549 and H23 cells, suggesting that apoptosis induced by CINK4 treatment is also associated with Rb activation.

Apoptosis in cancer has been of particular interest from a therapeutic standpoint, since therapies should ultimately lead to the eradication of cancer cells through apoptosis.[37] We were surprised to observe the synergistic anti-proliferative activity of paclitaxel simultaneously combined with CINK4, especially more than 10 μM concentration (>IC_{50}). This result be associated with potent apoptosis induced by higher concentration of CINK4. Apoptotic induction is a key mechanism for this combination in KRAS mutated adenocarcinoma with active Rb. In addition, the synergistic anti-proliferative activity also occurred

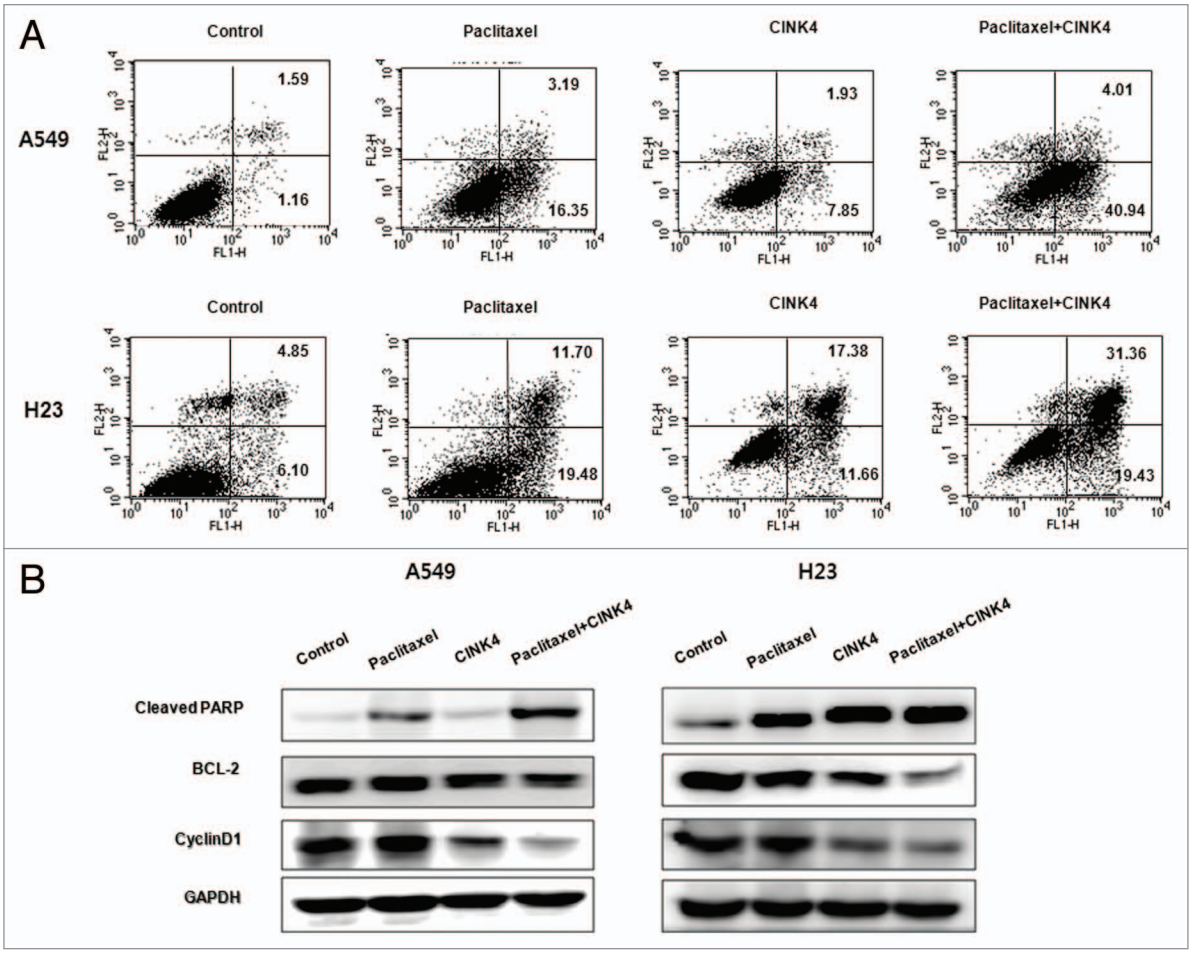

Figure 7. Paclitaxel combined with CINK4 increases apoptosis in A549 and H23 cells. (**A**) Apoptosis induction. Annexin V-FITC/PI double staining was performed. Annexin V-positive apoptotic cells were counted by flow cytometry. Each experiment was performed in triplicate. The typical apoptosis induction by single and combination treatments are shown. (**B**) Apoptosis-related protein expression. Actin was used as a loading control. Cells were treated for 72 h with paclitaxel (3 nM) and CINK4 (10 μM), alone or in combination.

by this combination in KRAS wild type PC14 cells (**Fig. S1**). It's suggested that the combination therapy is generally for tumor cells regardless of KRAS.

Alterations in the expression of genes that control the cell cycle may be of critical importance in determining drug sensitivity to anticancer agents. Cyclin D1 has been proposed as a chemoprevention target and a surrogate marker of chemopreventive response in lung cancer.[38,39] Targeting of cyclin D1 suppresses tumor growth via cell cycle arrest and promotes tumor apoptosis in NSCLC.[40-42] Inhibition of cyclin D1 leads to cellular apoptosis through stimulation of the NFκB pathway with nucleolar translocation of RelA.[43,44] Basseres et al. showed that deletion of RelA reduced the number of KRAS-mutant induced lung tumors with a higher number of apoptotic cells.[45] Therefore, our results suggest cyclin D1 plays a key role in apoptotic induction by CINK4 treatment in KRAS-mutated lung cancer.

The downregulation of Bcl-2 is thought to be one of the important modes of apoptosis induced by treatment with taxanes.[46] We observed that paclitaxel induces apoptosis, even in the absence of the reduction in Bcl-2 expression, suggesting that it may be attributed to the lower concentration of paclitaxel.[47] Our experiment demonstrated that CINK4 alone did not reduce Bcl-2 expression, but when combined with paclitaxel, Bcl-2 expression was significantly reduced. Paclitaxel alone did not reduce cyclin D1, but greatly reduced it when combined with CINK4. Our results suggest both Bcl-2 and cyclin D1 are important molecules for synergistic anti-proliferative activity when paclitaxel is combined with CINK4. We propose that downregulation of cyclin D1 by CINK4 may enhance sensitivity to paclitaxel, subsequently leading to increased apoptosis in KRAS-mutated adenocarcinoma cells.

In summary, our preclinical study demonstrated that genetic knockdown or pharmacological inhibition of CDK4 reduced the population of phosphorylated Rb and induced G_1 arrest. We also found the CDK4/6 inhibitor CINK4 promotes apoptotic induction through the downregulation of Rb and cyclin D1. In addition, cyclin D1 and Bcl-2 play critical roles in synergistic antitumor efficacy with significant apoptotic induction by combined treatment with CINK4 and paclitaxel in adenocarcinoma cells harboring KRAS mutations. We suggest CDK4 inhibition combined with taxane therapy may be a clinically useful strategy for KRAS mutant lung cancers.

Material and Methods

Drug preparation. The CDK4/6 inhibitor (CINK4) was purchased from EMD Biosciences and paclitaxel was kindly provided by Hanmi Pharmaceuticals. For in vitro experiments, CINK4 and paclitaxel were dissolved in 100% DMSO (Sigma) and diluted with culture medium to the desired concentration with a final DMSO concentration of 0.1%. DMSO was added to untreated cultures at 0.1% (v/v) as a solvent control.

Cancer cell lines. NSCLC cell lines A549 ($KRAS^{G12S}$), H358 ($KRAS^{G12C}$), SKLU-1 ($KRAS^{G12S}$), H23 ($KRAS^{G12C}$), and PC14 ($KRAS^{wild}$) were obtained from the Korea Cell Line Bank. H358 and H23 cells carry a wild-type CDKN2A (p16) gene, but A549, SKLU-1, and PC14 carry homozygous deletions of the CDKN2A gene. The somatic mutation data for KRAS and CDKN2A were obtained from the COSMIC database (www.sanger.ac.uk/cosmic).[16,48] A549 and SKLU-1 were propagated in Dulbecco's modified Eagle medium (DMEM, Gibco BRL). H358, H23, and PC14 cells were maintained in RPMI 1640 medium (WelGENE). Culture media were supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 1% penicillin-streptomycin (Gibco BRL) and 10 mmol/L HEPES (Amresco). Cells were cultivated in 100-mm plastic dishes (TPP) at 37 °C in 5% CO_2 and 95% air and harvested with trypsin/EDTA at logarithmic growth.

siRNA transfection and drug sensitivity assay. The day before siRNA transfection, 200 000 and 6000 cells were plated in 6- and 96-well plates, respectively and incubated in antibiotic-free medium with 10% FBS. After 24 h, the medium was exchanged with serum- and antibiotic-free medium. Lipofectamine 2000 (Invitrogen) was used to transfect CDK4 siRNA or universal negative control siRNA (Sigma-Aldrich) into cells. Both on-target siRNA and negative control siRNA were used at the same concentration in all experiments. The efficiency of inhibition was determined by reverse-transcription polymerase chain reaction (RT-PCR) and western blotting after 48 h transfection. Twenty-four hours after siRNA transfection, cytotoxic activity was measured during 48 h incubation with paclitaxel. Cytotoxicity was normalized to control siRNA-transfected, drug-treated cultures.

RT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen). cDNA was obtained with the Maxime RT premix kit (Intron). The following sense and antisense primers were used to amplify CDK4 (5'-CATCGTTCAC CGAGATCTGA-3' and 5'-CCAACACTCC ACATGTCCAC-3', 198-bp product) and GAPDH (5'-ACCCACTCCT CCACCTTTGA-3' and 5'-TCCAGGGGTC TTACTCCTTG-3', 147-bp product). Reaction conditions included a pre-denaturation step at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min, and extension for 5 min at 72 °C. PCR products were separated on 1.5% agarose gels.

Cell proliferation assay. Cells were seeded at 2000 per well in 96-well sterile plastic dishes (Nonclon), allowed to attach for 24 h and then treated with single-drug dilutions. The cells were treated with a series of final concentrations ranging from 0.1 to 40 μM for CINK4 and from 0.1 to 300 nM for paclitaxel. Inhibition of cell growth was measured with the sulforhodamine B (SRB) assay.[49] Absorbance was measured at 560 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Spectra Max 250; Molecular Devices). The drug concentrations required to inhibit cell growth by 50% (IC_{50}) were determined from dose-response curves created with Sigma Plot software (version 8.0). The results were presented as the mean ± standard deviation (SD) of at least 3 independent experiments.

Cell cycle analysis. Cells were seeded at 100 000 in 6-well plates (Nonclon) and incubated for 24 h. Then, each cell line was treated with CINK4 alone at 3, 5 and 10 μM for 48 h. After the treatments were completed, cells were trypsinized, washed twice with PBS and harvested by centrifugation. Briefly, cells were fixed with ice-cold 70% ethanol for at least 1 h, centrifuged, washed twice in cold PBS, resuspended in 1 mL PBS and stained with propidium iodide (PI) solution (0.05 mg/mL PI, 10 mg/mL RNase A) for 20 min at 37 °C in the dark. Fluorescence intensity was measured by flow cytometry (BD FACS Calibur); at least 10 000 cells were counted and DNA content analysis was performed with CellQuest software (Becton Dickinson). All experiments were performed in triplicate.

Apoptosis analysis. Cells were seeded in 6-well plates (Nonclon) at a density of 100 000 cells per well and incubated for 24 h prior to drug addition. After treatment, adherent and non-adherent cells were harvested and the FITC Annexin V Apoptosis Detection Kit I (BD PharMingen) was used according to the manufacturer's protocol. Briefly, cells were washed with cold PBS, resuspended in 1× binding buffer (0.1 M HEPES/NaOH, pH 7.4, 1.4 M NaCl, and 25 mM $CaCl_2$), stained with annexin V-FITC and PI, incubated for 15 min at room temperature in the dark and analyzed by flow cytometry (BD FACS Calibur). Cells unstained with PI and annexin V were considered normal (i.e., no apoptosis). Cells stained with annexin V alone or both annexin V and PI were considered to have undergone early and late apoptosis, respectively; these cells were included in the total count of apoptotic cells. The PI-positive and annexin V-negative cells were counted as necrotic cells. All experiments were performed in triplicate.

Western blot analysis. Cells were seeded at 500 000 in 100-mm plastic dishes and incubated for 24 h prior to addition of drug. After drug treatment, cells were harvested with trypsin/EDTA, washed twice with PBS and lysed with RIPA cell lysis buffer (Gibco BRL) containing a protease inhibitor cocktail (Amresco). Thirty micrograms of protein from each cell treatment was used in Bio-Rad detergent-compatible protein assays (Bio-Rad); proteins were resolved on 8–12% polyacrylamide gels by standard sodium dodecyl sulfate PAGE (SDS-PAGE) and transferred onto PVDF membranes (0.45 μm; Millipore). Membranes were blocked with 5% skim milk (Becton Dickinson). Blots were probed with the following antibodies. Anti-rabbit antibodies were used against Rb, phospho-Rb ($Ser^{807/811}$), $p16^{INK4A}$, phospho-ERK1/2 (Thr^{202}/Thr^{204}), cleaved caspase3 and cleaved PARP (Cell Signaling). Anti-mouse antibodies were used for CDK4, cyclin D1, Bax (Santa Cruz); CDK6 (Cell Signaling); actin and GAPDH (Abcam). Anti-goat antibodies were used against Bcl-2 (Santa Cruz). Horseradish peroxidase (HRP)-conjugated

secondary antibodies were purchased from Santa Cruz. Blots were developed with an enhanced chemiluminescence reagent (Amersham) and detected with an LAS 3000 Image analyzer system (Fujifilm).

Combined drug efficacy. We chose the drug concentrations and treatment times based on the results of single drug treatment; 4 concentrations of paclitaxel (1, 3, 5, and 10 nM) were combined with 4 concentrations of CINK4 (1, 3, 5, and 10 μM) in 3 cell lines over 72 h. The control wells consisted of cells incubated with culture medium. Interactions between paclitaxel and CINK4 were calculated by the combination index (CI). The calculated CI was then divided into 3 categories: CI < 1, CI = 1, or CI > 1, indicating synergistic, additive and antagonistic effects.[50]

Statistical analysis. Data are expressed as the mean ± SD. Statistically significant differences between groups were determined by the Student *t* test. In each case, P values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cbt/articles/24592

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