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Original Article

Palbociclib, a selective CDK4/6 inhibitor, enhances the effect of selumetinib in *RAS*-driven non-small cell lung cancer

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ABSTRACT

KRAS is one of the most commonly mutated oncogenes in non-small cell lung cancer (NSCLC). Resistance to MEK inhibitor monotherapy develops through a variety of mechanisms. *CDK4* was reported to have a synthetic lethal interaction with *KRAS*. In this study, we demonstrated the combination effects of the MEK inhibitor selumetinib and the CDK4/6 inhibitor palbociclib in *RAS*-driven NSCLC. In vitro, cell lines with *CDKN2A* mutations were insensitive to selumetinib. We used siRNA and pharmacologic inhibition of CDK4 and found that the combination of selumetinib and palbociclib synergistically inhibited *RAS*-driven NSCLC cases with *CDKN2A* mutations but not those with wild type *CDKN2A*. The combination treatment potentiated growth inhibition and increased the population of cells in G1 phase. Selumetinib completely inhibited p-ERK but not p-RB. The addition of palbociclib markedly inhibited p-RB and downregulated survivin expression. In vivo, the combination treatment inhibited the growth of NSCLC xenografts, which correlated with decreased levels of p-RB, downregulated survivin and decreased Ki-67 staining. These data suggest that the combination treatment of palbociclib and selumetinib is effective in preclinical models of *RAS*-driven NSCLC with *CDKN2A* mutations.

Introduction

Rat sarcoma virus (*Ras*) genes encode four highly related protein isoforms: *HRAS, KRAS4a, KRAS4b, and NRAS* [1,2]. *KRAS* is one of the most frequently mutated genes in NSCLC, which comprises approximately 30% of lung adenocarcinomas in Western populations and 11% in Asian populations [3,4]. However, at present, therapeutic targeting of RAS hasn't been demonstrated to be clinically feasible. Strategies that target single effector molecules downstream have also failed because multiple critical effectors such as the MEK-ERK, PI3K-AKT, and NF-kB pathways are activated [5].

Selumetinib is an orally active MEK inhibitor that inhibits ERK phosphorylation in many types of cancer cells including NSCLC-derived cells [6]. Although selumetinib has shown antitumor activity in vitro and in several tumor xenograft models, it has not demonstrated significant clinical activity in NSCLC when used as a single agent. Multiple mechanisms have been proposed to explain the failure of selumetinib in a monotherapy setting, including activation of PI3K-AKT [7]. Major efforts have been expended to combine MEK and PI3K pathway inhibitors in the clinic, but no drugs have been approved [8]. Thus, alternative strategies were needed to provide clinical benefits to different genetic subtypes of NSCLC.

The cyclin D/CDK4/RB signaling pathway is commonly disrupted in lung cancer. CDK4 protein expression in lung cancer is inversely correlated with the overall survival of patients [9]. Previous studies have reported a synthetic lethal interaction between *KRAS* and *CDK4* in a mouse model of NSCLC [10,11]. Furthermore, combined inhibition of MEK and CDK4 was found to elicit significant synergy in *RAS*-driven melanoma and colorectal cancer [8,12].

In this study, we investigated the efficacy of the combination of the MEK inhibitor selumetinib and the CDK4/6 inhibitor palbociclib in *RAS*-driven NSCLC and observed that this combination resulted in enhanced antitumor activity in cases with *CDKN2A* mutations both in vitro and in vivo.

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Table 1
Primer sequences used for real-time PCR.

mRNA	Oligonucleotides (5′ to 3′)
BIRC5(Survivin)-F	5-AGGACCACCGCATCTCTACAT-3
BIRC5(Survivin)-R	5-AAGTCTGGCTCGTTCTCAGTG-3
GAPDH-F	5-GACGCCATCAACACCGAGTT-3
GAPDH-R	5-CTTTGTCGTTGGTTAGCTGGT-3

Materials and methods

Cell lines and cell cultures

Human NSCLC cell lines, including A549, H23, H460, and H1299 cells, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). H23 cells carry a wild-type *CDKN2A* gene, while A549, H460 and H1299 cells carry homozygous deletions/mutations in the *CDKN2A* gene. The genetic alterations present in these cells are listed in Supplementary Table 1. A549, H460, and H23 cells were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Clark Bioscience, Houston, Texas, USA), at 37 °C in a 5% CO2 humidified atmosphere. H1299 cells were maintained in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% FBS, as described above.

Chemicals and antibodies

Selumetinib (AZD6244) was purchased from Selleck Chemicals. Palbociclib (PD0332991) was provided by Pfizer. For cellular studies, drugs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mM, and stock solutions were stored at −20 °C until further use. Antibodies against p-ERK1/2 (Thr202/Thy204), ERK1/2, p-RB (ser780), RB, survivin, and cyclin D1 were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against CDK4 was obtained from Abcam (Cambridge, MA, USA). Antibodies against GAPDH and β-actin were purchased from Boster Biological Technology (Wuhan, China). All siRNAs were obtained from GenePharma (Shanghai, China) and were used according to the manufacturer's instructions.

Cell viability assay and drug combination study

Cells were cultured in a 96-well plate at a density of 1000–2000 cells/well and allowed to attach for 24 h, which was followed by the addition of growth media containing serial dilutions of selumetinib, palbociclib, or both drugs in combination for 72 h. Cell viability was measured using a Cell Counting Kit 8 (Dojindo Laboratories, Tokyo, Japan). Combination index (CI) data were obtained using CompuSyn software (ComboSyn, Inc.). A CI of <1 was synergistic.

Colony formation assay

Cells were seeded in 6-well plates at a density of 100–500 cells/well. After adhesion, the cells were treated with DMSO (0.1%), selumetinib (1 μM), palbociclib (1 μM) or a combination of the two compounds. Moreover, the culture medium was refreshed every three days. After 10–14 days, the cells were stained with crystal violet and imaged.

siRNA transfection and drug sensitivity assay

The medium was exchanged for serum- and antibiotic-free medium during transfection. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect on-target siRNAs or negative control siRNA into the cells. 48 h after transfection, western blot was performed to determine the efficiency of inhibition. 24 h after CDK4 siRNA transfection, cytotoxic activity was measured during a 48-h incubation with selumetinib.

Cell cycle analysis

Cells were starved for 24 h before treatment with either DMSO (0.1%), selumetinib (1 μM), palbociclib (1 μM) or both drugs in combination. After treatment for 24 h, the cells were harvested, washed with PBS, fixed in 70% ethanol and stored at −20 °C for at least 24 h. Once they were prepared for analysis, the fixed cells were washed and resuspended in PBS, incubated with RNase and propidium iodide solution for 30 min at room temperature in the dark, and then analyzed using a BD FACSVerse (BD Biosciences, San Jose, CA, USA). The data were analyzed using FlowJo software Version 10.1.

Apoptosis analysis

After treatment for 48 h, the cells were harvested and apoptosis was detected using an FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, NJ, USA) according to the manufacturer's protocols. The cells were washed, resuspended in binding buffer and stained with 5 μl of FITC Annexin V and 5 μl of propidium iodide followed by incubation for 15 min in the dark at room temperature. Finally, apoptosis was analyzed by BD FACSVerse (BD Biosciences, San Jose, CA, USA).

Quantitative real-time PCR

The cells were treated for 48 h before RNA was extracted with TRIzol reagent (Biosharp). The cellular RNA concentration was measured using a NanoDrop 2000

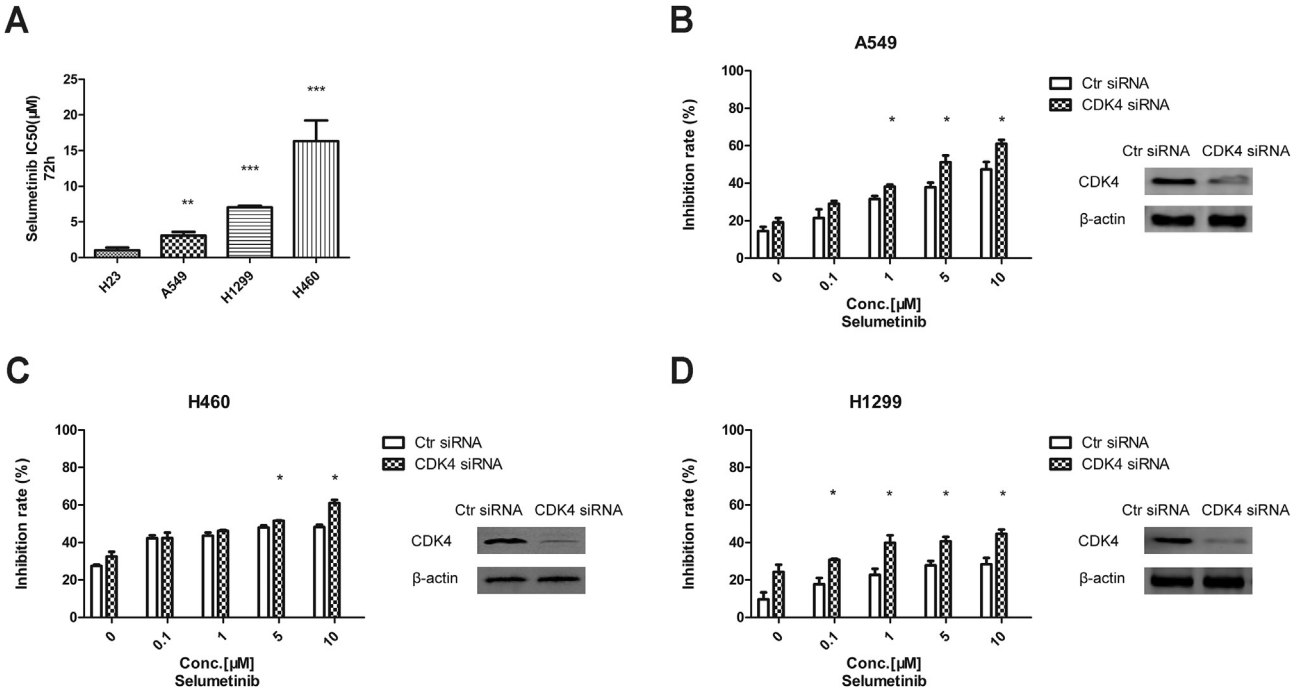

Fig. 1. *Ras*-driven NSCLC cell lines display varied sensitivities to selumetinib (A) and CDK4 knockdown enhances sensitivity to selumetinib (B, C, D). (A) Cell lines were seeded in 96-well plates and were treated with selumetinib at doses that ranged from 0.01 to 500 μM for 72 h; the IC50 values were then calculated. Data from three independent experiments represent the mean ± SD, $^{**}P < 0.01$; $^{***}P < 0.001$ vs H23 cells. (B, C, D) Enhanced anti-proliferative effect of selumetinib in CDK4 siRNA-transfected A549, H460 and H1299 cells. Data are presented as mean ± SD of triplicate experiments. $^{*}P < 0.05$ compared with control-transfected cells.