DCLAK11 treatment induces apoptosis in non-small cell lung cancer cells by inhibiting the mitochondrial apoptosis pathway

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Funding information
Natural Science Foundation of Inner Mongolia Autonomous Region

Abstract

Objective: DCLAK11 is a novel small molecule tyrosine kinase inhibitor with remarkable effects on cell proliferation and apoptosis in non-small cell lung cancer cells. Therefore, it is necessary to determine the molecular mechanisms of action of this compound. In this study, we investigated the antitumor effect that DCLAK11 exerts through inhibition of the mitochondrial apoptosis pathway.

Methods: Cell viability was assessed using the Cell Counting Kit-8 assay, and apoptosis was measured by an Annexin V-PI assay. The mitochondrial transmembrane potential was examined using JC-1 dye. Alterations in expression of the Bcl-2 family proteins Bcl-xL, Bax, Bid, Bim, Bad, and cleaved Caspase-9 were detected by western blotting.

Results: DCLAK11 inhibited the proliferation of non-small cell lung cancer cells HCC827 (IC50 = 10 nmol/L) and HCC4006 (IC50 = 20 nmol/L) in a dose-dependent manner. The Annexin V-PI assay results showed that DCLAK11 also significantly promoted apoptosis in both HCC827 and HCC4006 cells in a dose-dependent manner (F = 29.990, P < 0.00; F = 46.439, P < 0.001, respectively). The JC-1 assay results showed that the proportion of depolarized cells significantly increased with increasing concentrations of DCLAK11 when compared with that in the control HCC827 and HCC4006 cells (F = 63.910, P < 0.001; F = 11.831, P = 0.001, respectively). Western blot results showed that DCLAK11 caused a decrease in the expression level of Bcl-xL and an increase in the expression of Bid, Bim, Bad, Bax, and cleaved caspase-9 in a dose-dependent manner. Furthermore, the proportion of Bcl-xL/Bax decreased with increasing concentrations of DCLAK11 in HCC827 and HCC4006 cells.

Conclusion: We confirmed that DCLAK11 exerts an antitumor effect by inhibiting the mitochondrial apoptosis pathway.

KEYWORDS
apoptosis, DCLAK11, mitochondrial pathway, non-small cell lung cancer

1 | INTRODUCTION

Lung cancer is the most prevalent malignancy worldwide and is the leading cause of cancer-related death.1,2 Non-small cell lung cancer (NSCLC) is the main type of lung cancer,3 and chemotherapy is an important therapeutic strategy for patients at an advanced stage of this disease.4 However, the relatively serious side-effects of cytotoxic chemotherapy limit its benefits, and the overall survival of patients with NSCLC undergoing targeted therapies is far from satisfactory.5 Consequently, more effective treatments are urgently required for patients with NSCLC.

DCLAK11 is a novel small molecule tyrosine kinase inhibitor that shows remarkable antitumor activity in NSCLC.6 In the present study, we aimed to investigate the effects and mechanisms of action of DCLAK11 in inducing apoptosis in the NSCLC cell lines, HCC827 and HCC4006.
2 | METHODS

2.1 | Cell culture

The NSCLC cell lines, HCC827 and HCC4006, were obtained from the Institute of Biochemistry, and Cell Biology, Chinese Academy of Sciences (Shanghai, China), respectively. The cells were cultured in RPMI 1640 medium (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone). DCLAK11 was provided by Professor Hong Liu’s laboratory, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China.

2.2 | Cell Counting Kit-8 assay

Cells in the exponential phase were seeded into 96-well culture plates overnight and incubated with various concentrations of DCLAK11 for 72 h. Then, 10 µL of Cell Counting Kit-8 (CCK-8) solution (Dojindo, Shanghai, China) was added to the wells followed by incubation for 2–4 h. The absorbance was measured at 450 nm using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability (%) was calculated using the following equation: \( \frac{OD_{\text{treatment}}}{OD_{\text{control}}} \times 100\% = \text{cell viability} \% \).

2.3 | Annexin V-PI assay

The Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme Biotech, Nanjing, China) was used to determine the proportion of apoptotic HCC827 and HCC4006 cells. The assay was carried out according to the manufacturer’s protocol. Briefly, the cells were lysed in pancreatin without EDTA after incubation with various concentrations of DCLAK11 for 48 h. Cells collected in centrifuge tubes were washed by centrifugation in pre-cooled PBS (4°C, 300 g, 5 min, \( R = 9 \) cm) twice and resuspended in 100 µL 1X binding buffer before incubation with 5 µL Annexin V-FITC and 5 µL PI. The cells were then incubated for 10 min at room temperature in the dark. After incubation, the cells were resuspended in 400 µL 1X binding buffer. Apoptotic cells were measured by FACsCalibur (BD Bioscience, San Jose, CA, USA), and the resultant data were analyzed with FlowJo software (BD Bioscience).

2.4 | JC-1 assay

The HCC827 and HCC4006 cells were dissociated in pancreatin without EDTA after being incubated with various concentrations of DCLAK11 for 24 h. Approximately 11 × 10^5 HCC4006 cells and 6 × 10^5 HCC827 cells were resuspended in 0.5 mL RPMI 1640 medium and incubated in 0.5 mL JC-1 (Keygen Biotech, Nanjing, China) for 20 min in an incubator at 37°C. After centrifugation (4°C, 300 g, 3 - 4 min, \( R = 9 \) cm), the supernatants were discarded, and the cells were washed by centrifugation (4°C, 300 g, 3 - 4 min, \( R = 9 \) cm) in 1 mL 1X JC-1 staining buffer twice. Finally, the stained samples, resuspended in 500 µL 1X JC-1 staining buffer, were examined within 30 min with FACsCalibur (BD Bioscience).

2.5 | Antibodies and western blotting

Antibodies against caspase-3, cleaved caspase-8, cleaved caspase-9, Bim, Bad, Bid, Bcl-xL, Bax, and PARP were obtained from Cell Signaling Technology (Danvers, MA, USA). The actin antibody was obtained from Sigma Aldrich (St Louis, MO, USA), and the anti-rabbit immunoglobulin G and anti-mouse immunoglobulin G antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

HCC827 and HCC4006 cells were exposed to various concentrations of DCLAK11 for 48 h, and then lysed in RIPA buffer for 10–15 min. A bicinechonic acid assay was used to quantify the total protein concentration after cytolysis. The samples were then heated to 99°C for 10 min and resolved by 10–15% sodium dodecyl sulphate polyacrylamide gel electrophoresis and the proteins finally transferred to PVDF membranes. The membranes were first incubated with 3% bovine serum albumin at room temperature for 1 h, and subsequently probed with primary antibodies (1:1000) at 4°C overnight. The membranes were washed three times with Tris-buffered saline with Tween 20 for 10 min each, then immunoblotted with horseradish peroxidase-conjugated secondary antibodies (1:5000) at room temperature for 1 h. The membranes were again washed three times with Tris-buffered saline with Tween 20 for 10 min each, and the immunoreactive proteins were detected using ECL detection reagent (Thermo Fisher Scientific, Rockford, IL, USA). Images were captured with ImageQuant LAS 4000 (GE Healthcare, Chalfont St. Giles, UK).

2.6 | Statistical analysis

SPSS 19.0 software (SPSS, Chicago, IL, USA) was used for the statistical analyses. All quantitative data are presented as mean ± SD, \( n = 3 \). The data for multiple groups were compared using a one-way ANOVA. Data of two given groups were compared using a Dunnett’s test. The size of the test was \( \alpha = 0.05 \). A P-value <0.05 (double sided) was considered statistically significant.

FIGURE 1  Growth inhibition of HCC827 and HCC4006 cells on treatment with DCLAK11
FIGURE 2  DCLAK11 treatment induces apoptosis in HCC827 and HCC4006 cells. (a) HCC827 cells. (b) HCC4006 cells; $^a P < 0.01$ when compared with control; $^b P < 0.001$ when compared with control; $^c P < 0.05$ when compared with control.

FIGURE 3  DCLAK11 treatment decreases mitochondrial transmembrane potential ($\Delta\Psi_m$) in HCC827 and HCC4006 cells. (a) HCC827 cells were stained with JC-1. (b) HCC4006 cells were stained with JC-1. $^a P < 0.01$ when compared with control; $^b P < 0.001$ when compared with control; $^c P < 0.05$ when compared with control.
3 | RESULTS

3.1 | DCLAK11 treatment inhibits proliferation of NSCLC cells

We first investigated the viability of NSCLC cells on treatment with DCLAK11 (1, 3, 10, 30, 100, 1000 nmol/L), using the CCK-8 assay. DCLAK11 treatment markedly inhibited the proliferation of HCC827 and HCC4006 cells in a dose-dependent manner, with an IC50 value of 10 and 20 nmol/L, respectively (Fig. 1).

3.2 | DCLAK11 treatment induces apoptosis in NSCLC cells

HCC827 cells were treated with DCLAK11 at various concentrations (1, 3, 10, 30 nmol/L) for 48 h, after which the pro-apoptotic effect of DCLAK11 was quantified by Annexin V-FITC/PI double-staining and flow-cytometric analysis. As shown in Figure 2, the percentage of apoptotic cells among the HCC827 cells was significantly increased in a dose-dependent manner when compared with that in the untreated cells (1 nmol/L DCLAK11, 34.90 ± 6.22%; 3 nmol/L DCLAK11, 49.33 ± 10.42%; 10 nmol/L DCLAK11, 53.77 ± 4.55%; 30 nmol/L DCLAK11, 62.87 ± 3.19; control, 13.56 ± 2.70%; F = 29.990, P < 0.001).

We also detected the pro-apoptotic effect of DCLAK11 in the HCC4006 cell line. The cells were treated with DCLAK11 at various concentrations (3, 10, 30, 100 nmol/L) for 48 h. The percentage of apoptotic HCC4006 cells was similar to that of apoptotic HCC827 cells (3 nmol/L DCLAK11, 49.33 ± 10.42%; 10 nmol/L DCLAK11, 53.77 ± 4.55%; 30 nmol/L DCLAK11, 62.87 ± 3.19; control, 13.56 ± 2.70%; F = 29.990, P < 0.001). These data suggest DCLAK11 treatment significantly induces apoptosis in both HCC827 and HCC4006 cells (P < 0.001).
FIGURE 4 DCLAK11 treatment alters the expression of apoptotic proteins in HCC827 and HCC4006 cells. (a) Variations in the expression levels of apoptotic proteins were detected by western blotting. (b) Semiquantitative gray values of Bcl-xL/Bax

3.3 DCLAK11 treatment decreases mitochondrial transmembrane potential in NSCLC cells

As shown in Figure 3, after HCC827 and HCC4006 cells were treated with DCLAK11 for 24 h, damage to the mitochondrial membrane was observed. In HCC827 cells, the proportion of depolarized cells significantly increased from 4.30% to 27.34% with increasing doses of DCLAK11 when compared with that in the control ($F = 63.910$, $P < 0.001$). In HCC4006 cells, the proportion of depolarized cells significantly increased from 3.43% to 19.62% in a dose-dependent manner when compared with that in the control ($F = 11.831$, $P = 0.001$).

3.4 DCLAK11 treatment alters expression of apoptotic proteins

As shown in Figure 4, DCLAK11 decreased the expression levels of the anti-apoptotic protein, Bcl-xL, increased the expression of the pro-apoptotic Bcl-2 family proteins, Bid, Bim, Bad, and Bax, and induced cleavage of caspase-9 in a dose-dependent manner in both HCC827 and HCC4006 cells. Furthermore, the proportion of Bcl-xL/Bax also decreased with increasing concentrations of DCLAK11.

4 DISCUSSION

NSCLC is a malignant tumor that threatens human life, as it has a 5% 5-year survival rate and a median survival of $<$12 months when diagnosed at the extensive stage.5 Thus, a compelling need exists for the improved treatment of NSCLC to prolong the survival of patients with NSCLC.

Cell apoptosis plays an important role in balancing cell death and survival, and apoptotic dysfunction might induce carcinogenesis.7 The mitochondrial pathway, death receptor pathway, and endoplasm reticulum stress pathway are three major apoptotic pathways, among which the first is the most crucial.8 Apoptosis is reported to be regulated by the Bcl-2 family of proteins, which function upstream of irreversible cellular damage,9 and regulate the mitochondrial pathway by mediating mitochondrial outer membrane permeabilization.10 The Bcl-2 family proteins can be divided into two classes: anti-apoptotic, which includes Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bcl-B; and pro-apoptotic, which includes Bax, Bak, Bok, and the pro-apoptotic BH3-only proteins Bid, Bim, Bad, Bmf, Puma, Noxa, and Bik.11 In the presence of apoptotic stimuli, such as growth factor deprivation, DNA damage, oxidant stress, or nitric oxide (NO), the expression of Bax
and/or the BH3-only proteins Bad, Bid, and Bim increases, and they bind to Bcl-2/Bcl-xL to release Bax/Bak from inhibition. Free Bax and Bak form homo-oligomers, and insert stably into the outer mitochondrial membrane, resulting in increased mitochondrial outer membrane permeabilization, decreased ΔΨm, and release of cytochrome c into the cytoplasm. The released cytochrome c binds with the WD domain of apoptotic protease activating factor-1 (Apaf-1), leading to conformational changes in Apaf-1, followed by deoxyadenosine triphosphate (dATP) binding, and another conformational change in Apaf1 involving exposure and oligomerization of its CARD domain. This step is required for procaspase-9 binding to the exposed CARD domain and efficient assembly of the apoptosome, including cytochrome c, Apaf-1, and caspase-9. The apoptosome assembly results in cleavage of caspase-9, leading to production of the p37 and p19 subunits. Cleaved caspase-9 further processes caspase-3 and caspase-7, leading to apoptosis. Apoptotic dysfunction is closely related to tumor occurrence and development; hence, the research and development of novel antitumor drugs that can induce apoptosis has garnered increasing interest. Therefore, the present study aimed to examine the apoptosis-inducing effects of DCLAK11 through the mitochondrial pathway in NSCLC cell lines. First, our data confirmed that DCLAK11 inhibits proliferation and induces apoptosis in both HCC827 and HCC4006 cells. To elucidate the detailed mechanisms of apoptosis induced by DCLAK11 in the NSCLC cell lines, we identified important events and key proteins in the apoptosis pathway. We noted a decrease in the expression of the anti-apoptotic protein, Bcl-xL, and an increase in the expression of proapoptotic Bax and the BH3-only proteins, Bid, Bim, and Bad, and cleavage of caspase-9 on DCLAK11 treatment. The proportion of Bcl-xL/Bax establishes mitochondrial outer membrane permeabilization; hence their expression levels were measured semiquantitatively. As shown in Figure 4, the proportion of Bcl-xL/Bax decreased with increasing concentrations of DCLAK11 in HCC827 and HCC4006 cells. Damage to the ΔΨm is considered one of the earliest events in the apoptosis cascade. Once the mitochondrial potential collapses, apoptosis is irreversible. The present results showed that DCLAK11 induces mitochondrial membrane depolarization in HCC827 and HCC4006 cells in a dose-dependent manner.

In summary, the findings in the present study show that DCLAK11 induces apoptosis in HCC827 and HCC4006 cells through the mitochondrial pathway by inhibiting the expression of the anti-apoptotic Bcl-2 proteins, enhancing the expression of the proapoptotic Bcl-2 proteins, decreasing the proportion of Bcl-2/Bax, activating caspase-9, and inducing ΔΨm damage. Taken together, these data warrant further evaluation of the potential of DCLAK11 in the treatment of NSCLC.

CONFLICT OF INTEREST
The authors declare that they had read the article and there are no competing interests.

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How to cite this article: Guo X, Liu H, Ding J. DCLAK11 treatment induces apoptosis in non-small cell lung cancer cells by inhibiting the mitochondrial apoptosis pathway. Prec Radiat Oncol. 2019;3:94-99. https://doi.org/10.1002/pro6.1073