Biochemistry and Cell Biology

Ginsenoside metabolite compound K induces apoptosis and autophagy in non-small cell lung cancer cells via AMPK/mTOR and JNK pathways

Journal:	<i>Biochemistry and Cell Biology</i>
Manuscript ID	bcb-2018-0226.R1
Manuscript Type:	Article
Date Submitted by the Author:	04-Nov-2018
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Keyword:	Compound K, non-small cell lung cancer, apoptosis, autophagy, AMPK/mTOR
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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1 **Title page**

2 **Ginsenoside metabolite compound K induces apoptosis and**
3 **autophagy in non-small cell lung cancer cells via**
4 **AMPK/mTOR and JNK pathways**

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Abstract

Compound K (C-K, 20-O-(β -D-glucopyranosyl)-20(S)-protopanaxadiol), as a metabolite of ginsenoside, has been verified to have antitumor effects in various cancers, including non-small cell lung cancer (NSCLC). However, the detailed mechanisms of C-K in NSCLC remain largely unknown. In this study, we aimed to evaluate the effect of C-K on apoptosis and autophagy in NSCLC cells as well as its related mechanisms. According to the results, C-K suppressed the proliferation, led to G1 phase arrest and apoptosis in A549 and H1975 cells. Subsequently, C-K promoted autophagy as confirmed by enhanced rate of positive acridine orange staining cells, increased LC3II and Beclin-1 levels, while decreased p62 level in A549 and H1975 cells. Moreover, 3-Methyladenine (3-MA, an inhibitor of autophagy) effectively suppressed proliferation inhibition and apoptosis induced by C-K. Finally, C-K treatment promoted the activation of AMPK/mTOR and JNK signaling pathways. Treatment with compound C (AMPK inhibitor) or SP600125 (JNK inhibitor) significantly restrained C-K-induced proliferation inhibition, apoptosis and autophagy in A549 and H1975 cells. In conclusion, this study demonstrates that C-K promotes autophagy-mediated apoptosis in NSCLC via AMPK/mTOR and JNK signaling pathways.

Key words: Compound K, non-small cell lung cancer, apoptosis, autophagy, AMPK/mTOR

Introduction

Lung cancer is one of the most common and fateful malignant tumors worldwide(Siegel et al. 2017). Non-small cell lung cancer (NSCLC) accounts for 85 percent of all lung cancer cases(Ferlay et al. 2013). Most patients are diagnosed with advanced NSCLC and lose the operation chance(Carnio et al. 2014). Although remarkable progress has been made for surgery, radiotherapy and chemotherapy, the five-year survival of patients with lung cancer was only 20 percent(Safi et al. 2015). Exploring new chemotherapeutic agents has become one of hot spots in lung cancer at present. Notice that although some drugs have shown good anticancer effect, there is still a lack of the deep research of their mechanisms.

Compound K (C-K, 20-O-(β -D-glucopyranosyl)-20(S)-protopanaxadiol) is the main metabolite product of ginsenoside, and has been found to inhibit the proliferation in a variety of cancers(Lee et al. 1999; Zhou et al. 2006). The delivery and efficiency of C-K mixed with micelles was significantly improved, which enhanced the antitumour efficacy of C-K in lung cancer(Yang et al. 2016; Zhang et al. 2017). In addition, C-K combination with gamma-ray radiation or cisplatin enhanced the efficacy of radiotherapy and chemotherapy in lung cancer(Chae et al. 2009; Li et al. 2015). C-K promoted TRAIL-induced apoptosis via autophagy-mediated upregulation of DR5 in colon cancer cells(Chen et al. 2016). However, the detailed anticancer mechanisms of C-K in lung cancer are far from clear.

Autophagy is a biological process that maintains homeostasis via capturing and degrading impaired proteins and organelles. Autophagy has been recognized to have

both promoting and suppressing effects on cancer cells. It has been shown that the level of autophagy rises during hypoxia, which promotes the survival of cancer cells (Degenhardt et al. 2006). Studies also demonstrated that the enhanced autophagy contributed to cancer cell growth, invasion and metastasis (Guo et al. 2011; Lock et al. 2014). In contrast, plenty of studies suggested that inhibition of autophagy promoted the development of lung and liver cancers (Qu et al. 2003; Yue et al. 2003). Recent research found that modified regular ginseng extract (containing Rh2, Rg3, C-K and protopanaxatriol type) could induce autophagy in lung cancer cells (Yoo et al. 2017). In addition, C-K has been confirmed to induce autophagy and apoptosis in colon cancer cells and melanoma (Kang et al. 2014; Kim et al. 2013). In lung cancer cells, whether C-K can induce autophagy and its role in apoptosis has not been determined.

In the present study, for the first time, we investigated the role of C-K in the regulation of autophagy-mediated apoptosis of NSCLC cells, and also evaluated its related mechanisms.

Materials and Methods

Chemicals and reagents

C-K was purchased from Chengdu PureChem-Standard Co., Ltd and dissolved in DMSO. The Chemical structure of C-K is shown in Fig. 1(A). 3-MA, compound C, and SP600125 were purchased from MedChemExpress (USA). 3-MA was dissolved in deionized water. Compound C and SP600125 were dissolved in DMSO.

Cell lines and treatments

A549 and H1975 cell lines were obtained from Cell Bank of Shanghai Institute of

Chinese Academy of Sciences and cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (Hyclone, USA) in a humidified incubator with 5% CO₂ at 37 °C. A549 and H1975 cells were pre-treated with or without 2 mM 3-MA for 3 h, 10 μM compound C for 1 h, 20 μM SP600125 for 1 h, and then treated with various concentrations of C-K for 24 h.

MTT assay

MTT assay was performed to determine cell proliferation. A549 and H1975 cells (4×10^3 per well) were seeded into 96-well plates and received different treatments, with five replicates. Then cells were incubated with 0.5 mg/ml MTT for 4 h at 37 °C. After discarding the supernatant, 150 μl DMSO was added into each well. The absorbance at 570 nm was detected by a microplate reader (Bio-Tek, USA).

Flow cytometry for cell cycle analysis

Propidium iodide (PI) staining was adopted to evaluate cell cycle distribution. Briefly, A549 and H1975 cells from different treatment groups were collected, washed with cold PBS, and then fixed with cold 70% ethanol at 4°C for 2 h. After being washed with cold PBS, cells were incubated with staining buffer containing 25 μl PI and 10 μl RNase A (Beyotime, China) at 37°C for 30 min in the dark. Cell cycle was detected using flow cytometry (BD, USA).

Acridine orange staining

The production of acidic intracellular compartments is a specific process of autophagy, which was observed by acridine orange staining. After being subjected to various treatments, A549 and H1975 cells were fixed in 4% paraformaldehyde for 15 min,

washed with PBS for three times, and stained with 0.01% acridine orange (Solarbio, China) for 5 min at room temperature. Then the cells were washed with PBS, observed and photographed under a fluorescence microscope (Olympus, Japan) at a magnification of 200 X.

Flow cytometry for cell apoptosis analysis

Apoptosis of A549 and H1975 cells was assessed by Annexin V/PI staining. Briefly, A549 and H1975 cells were suffered to different treatments and collected, washed with PBS twice. Then the cells were incubated with 5 µl Annexin V-FITC and 5 µl PI in 500 µl Binding Buffer for 15 min at room temperature in the dark. This cells were detected by flow cytometry (BD, USA).

Western blot

The protein levels in A549 and H1975 cells were assessed by western blot assay. Cells from different groups were collected and lysed in RIPA buffer (Beyotime, China) containing 1% PMSF (Beyotime) on ice for 5 min. After centrifugation at 10,000 g for 10 min at 4°C, the cell supernatants were collected. Protein concentration was measured using an Enhanced BCA Protein Assay Kit (Beyotime). Protein samples (40 µg) were subjected onto SDS-PAGE and blotted to PVDF membranes (Millipore, USA). After blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated with primary antibodies LC3I/II (1:2000, Cell Signaling Technology, USA), p62 (1:1000, Cell Signaling Technology), Beclin-1 (1:1000, Cell Signaling Technology), cleaved caspase-3 (1:1000, Cell Signaling Technology), cleaved PARP (1:1000, Abcam, USA), p-AMPK (1:1000, Cell

Signaling Technology), AMPK (1:1000, Cell Signaling Technology), p-mTOR (1:1000, Cell Signaling Technology), mTOR (1:1000, Cell Signaling Technology), p-c-Jun (1:1000, Cell Signaling Technology), c-Jun (1:1000, Cell Signaling Technology), p-JNK1/2 (1:1000, Cell Signaling Technology), JNK1/2 (1:1000, Sangon, China), β -actin (1:5000, Bioss, China) at 4°C overnight. Then, the membranes were incubated with HRP-labeled Goat Anti-Rabbit IgG(H+L) (1:5000, Beyotime) at 37°C for 45 min. Blots were visualized using ECL Plus (Beyotime) and analyzed by Gel-Pro-Analyzer software.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD). For comparison among multiple groups, one-way analysis of variance followed by Newman-Keuls Multiple Comparison Test was performed by GraphPad Prism 5 software. Unpaired student's t-test was used to compare only two groups. *P* values less than 0.05 were considered statistically significant.

Results

C-K inhibited proliferation and induced apoptosis of NSCLC cells.

As shown in Fig. 1(B), the proliferation of A549 and H1975 cells was restrained by C-K in a concentration dependent manner as evaluated by MTT assay. In addition, cell cycle distribution was detected and presented in Fig. 1(C-E). C-K dose dependently increased the percentage of A549 and H1975 cells in G1 phase, while decreased the percentage in S and G2/M phases. Moreover, apoptosis rate of A549 and H1975 cells was enhanced with the increase of C-K concentration (Fig.

1(F)&(G)). These results suggested that the proliferation was inhibited and apoptosis of A549 and H1975 cells was promoted by C-K.

C-K induced autophagy of NSCLC cells.

To determine the autophagy level in A549 and H1975 cells, acridine orange staining was performed. As illustrated in Fig. 2 (A)&(B), the percentage of positive acridine orange staining cells was significantly raised by C-K treatment. Subsequently, the levels of autophagy-related proteins were assessed by western blot assay. As presented in Fig. 2(C-F), the ratio of LC3II/LC3I and protein level of Beclin-1 were upregulated, while p62 level was downregulated by C-K in A549 and H1975 cells. The above results indicated that autophagy was induced by C-K in NSCLC cells.

C-K promoted apoptosis of NSCLC cells via inducing autophagy.

Next, we investigated the relationship between apoptosis and autophagy induced by C-K. The A549 and H1975 cells were pre-treated with 3-MA, an inhibitor of autophagy. As shown in Fig. 3(A)&(B), C-K-induced positive acridine orange staining in A549 and H1975 cells was strikingly suppressed by 3-MA. Moreover, the enhanced ratio of LC3II/LC3I and protein level of Beclin-1, and decreased p62 level induced by C-K were restrained by 3-MA (Fig. 3(C-F)). As shown in Fig. 3(G), 3-MA significantly weakened the inhibiting effect of C-K on proliferation of A549 and H1975 cells. Moreover, C-K-induced apoptosis of A549 and H1975 cells was obviously repressed by 3-MA (Fig. 3(H)&(I)). As illustrated in Fig. 3(J-L), the apoptosis-related protein levels were detected. Treatment with C-K enhanced the cleaved caspase-3 and cleaved PARP levels in A549 and H1975 cells, which could be

restrained by 3-MA.

C-K induced autophagy-mediated apoptosis in NSCLC cells via regulating AMPK/mTOR signaling pathway

To evaluate the mechanisms through which C-K regulated autophagy in A549 and H1975 cells, we focused on AMPK/mTOR signaling pathway. As presented in Fig. 4(A-C), the ratio of p-AMPK/AMPK was increased and the ratio of p-mTOR/mTOR was decreased by C-K administration. To inactivate AMPK/mTOR signaling pathway, A549 and H1975 cells were pre-treated with an AMPK inhibitor, compound C. As illustrated in Fig. 4(D)&(E), the increased percentage of positive acridine orange staining cells induced by C-K was inhibited by compound C. C-K-induced inhibition of proliferation in A549 and H1975 cells was suppressed by compound C (Fig. 4(F)). Moreover, pre-treatment with compound C restrained C-K-induced apoptosis of A549 and H1975 cells (Fig. 4(G)&(H)). As shown in Fig. 4(I-L), the increased ratios of p-AMPK/AMPK and LC3II/LC3I, and decreased ratio of p-mTOR/mTOR after administration of C-K were inhibited by compound C. These results suggested that C-K induced autophagy of A549 and H1975 cells via regulating AMPK/mTOR signaling pathway.

C-K induced autophagy-mediated apoptosis in NSCLC cells via activating JNK signaling pathway

Further, as detected by western blot, the ratio of p-c-Jun/c-Jun in A549 and H1975 cells was raised by C-K (Fig. 5(A)&(B)). Moreover, A549 and H1975 cells were pre-treated with SP600125 to inhibit the activation of JNK pathway. As shown in Fig.

5(C)&(D), pre-treatment with SP600125 repressed C-K-induced the positive acridine orange staining in A549 and H1975 cells. The inhibition of proliferation in A549 and H1975 cells by C-K was suppressed by SP600125 (Fig. 5(E)). In addition, C-K-induced apoptosis was suppressed by pre-treatment with SP600125 (Fig. 5(F)&(G)). The increased ratios of p-JNK/JNK, p-c-Jun/c-Jun and LC3II/LC3I induced by C-K were restrained by SP600125 pre-treatment in A549 and H1975 cells (Fig. 5(H-K)). So C-K induced autophagy-mediated apoptosis in A549 and H1975 cells through activation of JNK pathway.

Discussion

C-K is the major metabolite of ginsenoside and has been shown to possess effective anti-cancer capabilities in a variety of tumors, including NSCLC(Yang et al. 2016). However, the anti-cancer mechanisms of C-K in NSCLC have not been fully understood. In this study, we investigated the effect of C-K on apoptosis and autophagy in NSCLC cells for the first time. Our results offered first evidence that C-K promoted apoptosis of NSCLC cells through inducing autophagy via AMPK/mTOR and JNK signaling pathways.

Firstly, we evaluated the anticancer effects of C-K through detecting its role in proliferation, cell cycle progression, and apoptosis in two NSCLC cell lines, A549 and H1975. Previous researches have demonstrated that C-K inhibited cancer cell growth via causing cell cycle arrest and apoptosis(Chen et al. 2013; Kwak et al. 2015; Zheng et al. 2014). Consistently, our results showed that C-K dose dependently inhibited proliferation, induced G1 cell cycle arrest and apoptosis in A549 and H1975

cells. These results strongly verified the anticancer effects of C-K on NSCLC cells.

Autophagy as a homeostatic process is responsible for degrading impaired proteins and organelles (Di Benedetto et al. 2017). Growing evidence has suggested that autophagy acts as a “double-edged sword” in both promoting and suppressing tumor development (Li et al. 2017; Yoshida 2017). So far, the effect of C-K on autophagy in NSCLC cells has not been reported. Herein, according to our results C-K induced autophagy in A549 and H1975 cells as evidenced by stronger acridine orange staining. To further confirm the role of C-K in autophagy, a series of autophagy-related proteins were evaluated. It has been recognized that LC3 serves crucial roles in autophagy and the forming of LC3-II is a marker for the formation of autophagosomes (Pan et al. 2015; Zou et al. 2012). Beclin-1 is also an important marker for autophagosomes, which is upregulated and regulates the ongoing autophagy (Zou et al. 2012). P62 is a protein degraded during autophagy and indicates autophagic flux (Hang et al. 2018). According to our results, C-K treatment resulted in increase in LC3-II/LC3I ratio and Beclin-1 level, and decrease in p62 level, which suggested that C-K induced autophagy in NSCLC cells.

It has been confirmed that autophagy facilitates cell death, and also cell survival in cancer cells (Guo et al. 2011; Wang et al. 2018). So the mechanisms of autophagy in cancer are complicated. In this study, we further explored the crosstalk between apoptosis and autophagy in C-K-treated NSCLC cells. In order to achieve that, 3-MA, an inhibitor of autophagy, was adopted. Our results indicated that 3-MA partially reversed the anticancer effects of C-K as confirmed by increased proliferation and

reduced apoptosis rate when compared with C-K treatment group. Therefore, we could draw a conclusion that C-K induced autophagic cell death in NSCLC cells.

To further elucidate the regulatory mechanisms of C-K in autophagy, we focused on AMPK/mTOR signaling pathway. AMPK is an AMP-dependent protein kinase that is a key molecule for adjustment of energy metabolism in eukaryotic cells. It is confirmed that activation of AMPK may promote the dephosphorylation of mTOR, which regulates cell growth and metabolism(Lin et al. 2013). Mounting evidence demonstrates that AMPK-mTOR signaling pathway participates in the regulation of autophagy. For example, Wang et al. suggested that berberine modulated autophagy in glioblastoma multiforme cells via targeting AMPK-mTOR pathway(Wang et al. 2016). In lung cancer, lycorine facilitated autophagy-mediated apoptosis in A549 cells via promoting AMPK phosphorylation and mTOR dephosphorylation(Zeng et al. 2017). In the present study, C-K treatment led to increased phosphorylation of AMPK, while decreased phosphorylation of mTOR in NSCLC cells. In addition, treatment with compound C, an inhibitor of AMPK, effectively inhibited C-K-induced autophagy and proliferation inhibition. Therefore, C-K induced autophagic death in NSCLC cells via regulating AMPK-mTOR pathway.

C-Jun is one of the target proteins of c-Jun N-terminal kinase (JNK), which has been reported to regulate apoptosis via affecting autophagy(Lorin et al. 2010). C-Jun, as a nuclear transcriptional regulatory factor, is usually phosphorylated by JNK(Galardi et al. 2011). According to our results, the phosphorylation of c-Jun was enhanced by C-K in NSCLC cells. Moreover, treatment with SP600125, an inhibitor

of JNK, significantly reversed C-K-induced autophagy and proliferation inhibition in NSCLC cells. So JNK pathway was also involved in C-K-induced autophagic death in NSCLC cells.

In summary, the present study found that C-K inhibited the proliferation, induced G1 phase arrest and apoptosis of NSCLC cells. Autophagy was obviously induced by C-K treatment, which mediated proliferation inhibition and apoptosis in NSCLC cells. C-K induced autophagy through activating AMPK/mTOR and JNK signaling pathways. Our study provides insights into the antitumor mechanisms of C-K, which provides more theoretical basis for C-K, as a promising strategy for NSCLC.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1 Effect of C-K on apoptosis of NSCLC cells. A549 and H1975 cells were treated with different concentrations of C-K (0, 20, 40, 60, and 80 µg/ml) for 24 h. (A) Chemical structure of C-K. (B) Cell viability was determined by MTT assay (n=5). (C) Cell cycle progression was assessed by flow cytometric analysis. The percentage of

422 A549 (D) and H1975 (E) cells in G1, S, G2/M phases was presented (n=3). (F)
423 Apoptosis of A549 and H1975 cells was detected by Annexin V/PI staining on flow
424 cytometry. (G) The apoptosis rate of A549 and H1975 cells was calculated and shown
425 (n=3). All data were presented as means $\pm$ SD. ***P < 0.001, versus the 0 μ g/ml
426 group.

427 **Figure 2** Effect of C-K on autophagy of NSCLC cells. (A) Autophagic lysosomes in
428 A549 and H1975 cells were detected by acridine orange staining at 24 h after
429 treatment with C-K. (B) The percentage of positive acridine orange staining cells was
430 quantified (n=3). (C) After treatment with C-K for 24 h, the protein levels of LC3I,
431 LC3II, p62, Beclin-1 in A549 and H1975 cells were assessed by western blot assay.
432 β -actin was used as a loading control. (D-F) The relative optical density of the protein
433 bands was quantified (n=3). All data were presented as means $\pm$ SD. ***P < 0.001,
434 versus the 0 μ g/ml group.

435 **Figure 3** Effect of autophagy on C-K-induced apoptosis in NSCLC cells. A549 and
436 H1975 cells were pre-treated with or without 2 mM 3-MA for 3 h, then treated with or
437 without 20 μ g/ml C-K for 24 h. (A) Autophagic lysosomes in A549 and H1975 cells
438 were detected by acridine orange staining. (B) The percentage of positive acridine
439 orange staining cells was quantified (n=3). (C) The protein levels of LC3I, LC3II, p62,
440 Beclin-1 in A549 and H1975 cells were assessed by western blot assay. β -actin was
441 used as a loading control. (D-F) The relative optical density of the protein bands was
442 quantified (n=3). (G) Cell viability of A549 and H1975 cells was determined by MTT
443 assay (n=5). (H)&(I) Apoptosis of A549 and H1975 cells was detected by Annexin

V/PI staining on flow cytometry and quantified (n=3). (J) The protein levels of cleaved caspase-3 and cleaved PARP in A549 and H1975 cells were assessed by western blot assay. β -actin was used as a loading control. (K-L) The relative optical density of the protein bands was quantified (n=3). All data were presented as means $\pm$ SD. ***P < 0.001, versus the control group. #P < 0.05, ##P < 0.01, ###P < 0.001, versus the C-K group.

Figure 4 Effect of AMPK/mTOR signaling pathway on C-K-induced autophagy in NSCLC cells. (A) The protein levels of p-AMPK, AMPK, p-mTOR, and mTOR in A549 and H1975 cells were assessed by western blot assay at 24 h after treatment with 20 μ g/ml C-K. β -actin was used as a loading control. (B-C) The relative optical density of the protein bands was quantified (n=3). A549 and H1975 cells were pre-treated with or without 10 μ M compound C for 1 h, then treated with or without 20 μ g/ml C-K for 24 h. (D-E) Autophagic lysosomes in A549 and H1975 cells were detected by acridine orange staining and quantified (n=3). (F) Cell viability was determined by MTT assay (n=5). (G)&(H) Apoptosis of A549 and H1975 cells was detected by Annexin V/PI staining on flow cytometry and quantified (n=3). (I) The protein levels of p-AMPK, AMPK, p-mTOR, mTOR, and LC3I/II were assessed by western blot assay. β -actin was used as a loading control. (J-L) The relative optical density of the protein bands was quantified (n=3). All data were presented as means $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001, versus the control group. #P < 0.05, ##P < 0.01, ###P < 0.001, versus the C-K group.

Figure 5 Effect of c-Jun signaling pathway on C-K-induced autophagy in NSCLC

466 cells. The protein levels of p-c-Jun and c-Jun in A549 and H1975 cells were assessed
467 by western blot assay at 24 h after treatment with 20 µg/ml C-K. β-actin was used as
468 a loading control. (B) The relative optical density of the protein bands was quantified
469 (n=3). A549 and H1975 cells were pre-treated with or without 20 µM SP600125 for 1
470 h, then treated with or without 20 µg/ml C-K for 24 h. (C-D) Autophagic lysosomes
471 in A549 and H1975 cells were detected by acridine orange staining and quantified
472 (n=3). (E) Cell viability was determined by MTT assay (n=5). (F)&(G) Apoptosis of
473 A549 and H1975 cells was detected by Annexin V/PI staining on flow cytometry and
474 quantified (n=3). (H) The protein levels of p-JNK1/2, JNK1/2, p-c-Jun, c-Jun, and
475 LC3I/II were assessed by western blot assay. β-actin was used as a loading control.
476 (I-K) The relative optical density of the protein bands was quantified (n=3). All data
477 were presented as means $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001, versus the
478 control group. #P < 0.05, ##P < 0.01, versus the C-K group.

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Figure 1

277x438mm (300 x 300 DPI)

209x228mm (300 x 300 DPI)

Figure 3

337x410mm (300 x 300 DPI)

Figure 4

294x456mm (300 x 300 DPI)

Figure 5

209x412mm (300 x 300 DPI)