

Article

Cryptotanshinone induces cell cycle arrest and apoptosis of NSCLC cells through the PI3K/Akt/GSK-3 β pathway.

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Abstract: Cryptotanshinone (CTT) is a natural product and a quinoid diterpene isolated from the root of the Asian medicinal plant, *Salvia miltiorrhiza bunge*. Notably, CTT has a variety of anti-cancer actions, including the activation of apoptosis, anti-proliferation, and a reduction in angiogenesis. We further investigated the anti-cancer effects of CTT in A549 and H460 which are NSCLC cell lines. CTT treatment in NSCLC cells reduced cell growth through PI3K/Akt/GSK3 β pathway inhibition, G0 / G1 cell cycle arrest, and the activation of apoptosis. CTT induced increase of Bax and cleavage of apoptosis-related signaling such as caspase-3, caspase-9, poly-ADP-ribose polymerase (PARP), and Bax, as well as inhibition of anti-apoptosis related signaling such as Bcl-2, survivin, and cellular-inhibitor of apoptosis protein 1 and 2 (cIAP-1 and -2). It also induced G0/G1 phase cell cycle arrest by decreasing the expression of cyclin A, cyclin D, cyclin E, Cdk 2, and Cdk 4. In addition, CTT reduced the protein expression of the PI3K/Akt/GSK3 β signaling pathway related to cell proliferation. These results highlight the latent potential of CTT as natural therapeutic agent for NSCLC.

Keywords: Cryptotanshinone; NSCLC; cell cycle arrest; apoptosis; PI3K/Akt/GSK3 β ;

1. Introduction

Lung cancer has 2 major subtypes: non-small-cell Lung cancer (NSCLC) and small-cell lung cancer (SCLC) [1]. Eighty to 85% of lung cancer is constituted as NSCLC but only 5% of patients with NSCLC survive after the fourth stage. NSCLC therapies, such as surgery, chemotherapy, irradiation, etc. have been used, but a better, more efficient therapy is still needed to cure NSCLC [2,3]. This is because only 15-30% of lung cancer patients are cured with chemotherapy and the symptoms of cancer can even return shortly after surgery due to the metastatic nature of the disease [4,5]. Therefore, a more efficient alternative agent for anti-cancer treatment is needed urgently.

Natural products have been shown to have fewer side effects and higher efficacies than conventional chemotherapy anticancer drugs [6]. Cryptotanshinone (CTT) is a natural product and a quinoid diterpene isolated from the root of the Asian medicinal plant, *Salvia miltiorrhiza bunge*. Published studies have reported the anti-inflammation [7], anti-diabetes [8], and anti-cancer effects of CTT. CTT has a variety of anti-cancer actions in particular, including anti-proliferation, activation of apoptosis, and a reduction in angiogenesis [9-11].

These days, representative chemotherapeutic agents used in NSCLC are epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) [12]. EGFR-TKIs therapies show considerable promise, but people who use docetaxel or cisplatin to treat EGFR antigens exhibit a drug resistance to EGFR-TKIs, and they also have the significant side effect of excessive cytotoxicity. These negative

side effects are clear in all EGFR-TKIs, but one of them, Gefitinib (GF), prolongs progression-free survival with acceptable toxicity, compared to other standard chemotherapy agents [13,14].

With the previously discovered efficacy of CTT, we hypothesized that CTT would be an effective anti-NSCLC agent. As a result, we tried in all experiments to confirm the possibility of CTT as an anti-NSCLC agent by comparing GF, which is a representative EGFR-TKI with low side effects and high efficacy.

Herein, our results demonstrated the anti-cancer effects of CTT in NSCLC showing G0/G1 cell cycle arrest, activation of apoptosis, and inhibition of cell proliferation through PI3K/Akt/GSK3 β cell signaling pathway. Therefore, our study provides the latent possibility of CTT as an NSCLC natural therapeutic agent.

2. Results

2.1. CTT inhibited the viability of A549 and H460 cells.

MTS assays were performed to evaluate the effects of CTT on the viability of A549 and H460 cells. As shown in Fig. 1A, CTT markedly decreased the viability of A549 and H460 cells in a dose- and time-dependent manner. Notably, the cytotoxic effects of 10 μ M CTT on NSCLC cells were higher than GF at both 24 h and 48 h. Furthermore, LDH release in the culture supernatant was increased in a dose-dependent manner after treatment of CTT for 24h (Fig. 1B). Thus, these results indicate that CTT inhibited the viability of A549 and H460 cells.

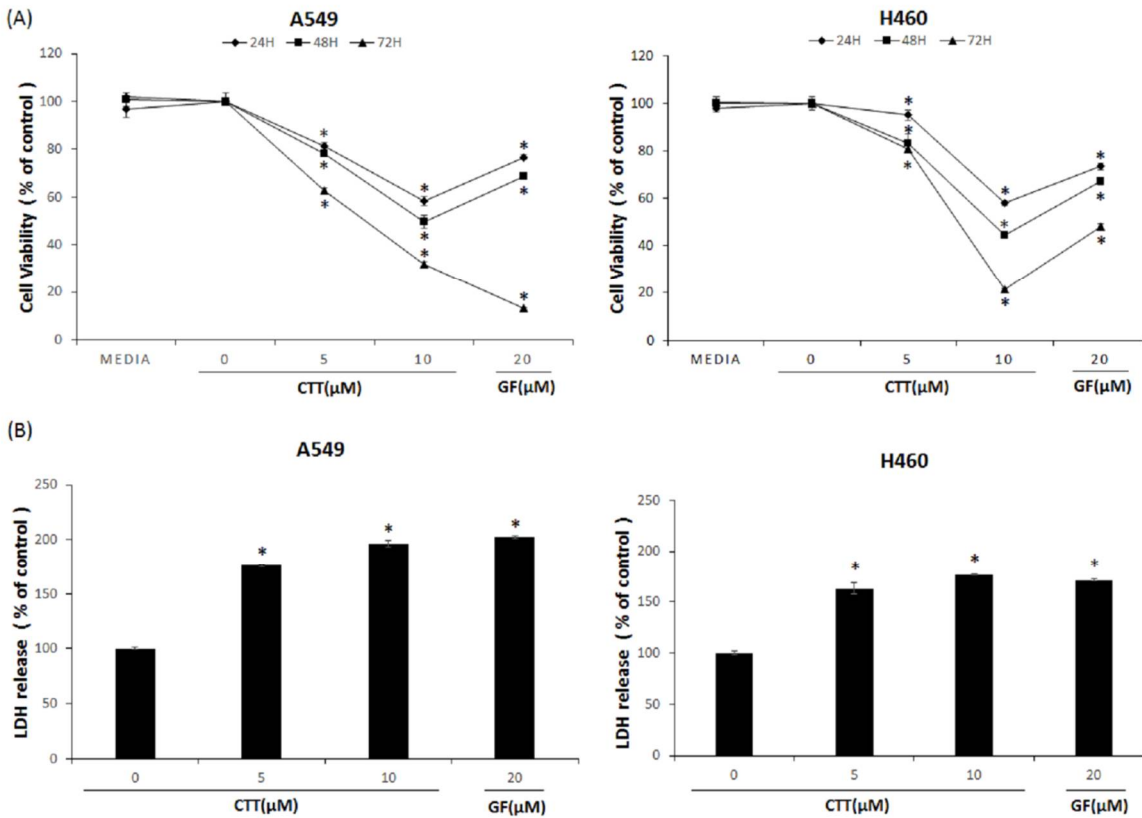


Figure 1. Effects of CTT treatment on cytotoxicity in A549 and H460 cells. (A) Cell viability was evaluated through MTS assay after 24, 48, and 72 h treatment with 0, 5, or 10 μ M of CTT or 20 μ M GF (clinical anticancer drug). *P < 0.05 compared to the control group. (B) LDH release was assessed using an LDH assay kit after 24 h treatment with 0, 5, or 10 μ M of CTT or 20 μ M GF (clinical anticancer drug). *P < 0.05 compared to the 0 μ M of CTT group. The data represent each of the three independent experiments. Significant differences for the treated groups were determined by Duncan's test for multiple comparisons. Values represented as mean \pm SD from each experiments.

2.2. CTT increased A549 and H460 apoptosis.

To confirm whether the effects of CTT on the cytotoxicity of NSCLC cells were related to apoptosis, an Annexin V assay was performed. As shown in Fig. 2 (A, B, D and E), CTT dose-dependently increased apoptosis in A549 and H460. The cells were stained with DAPI to better represent the obvious morphological changes related to apoptosis (Fig. 2 (C, F)). The white arrow markers show nuclear condensation and fragmentation. Thus, these results indicate that CTT induced cytotoxicity by apoptosis.

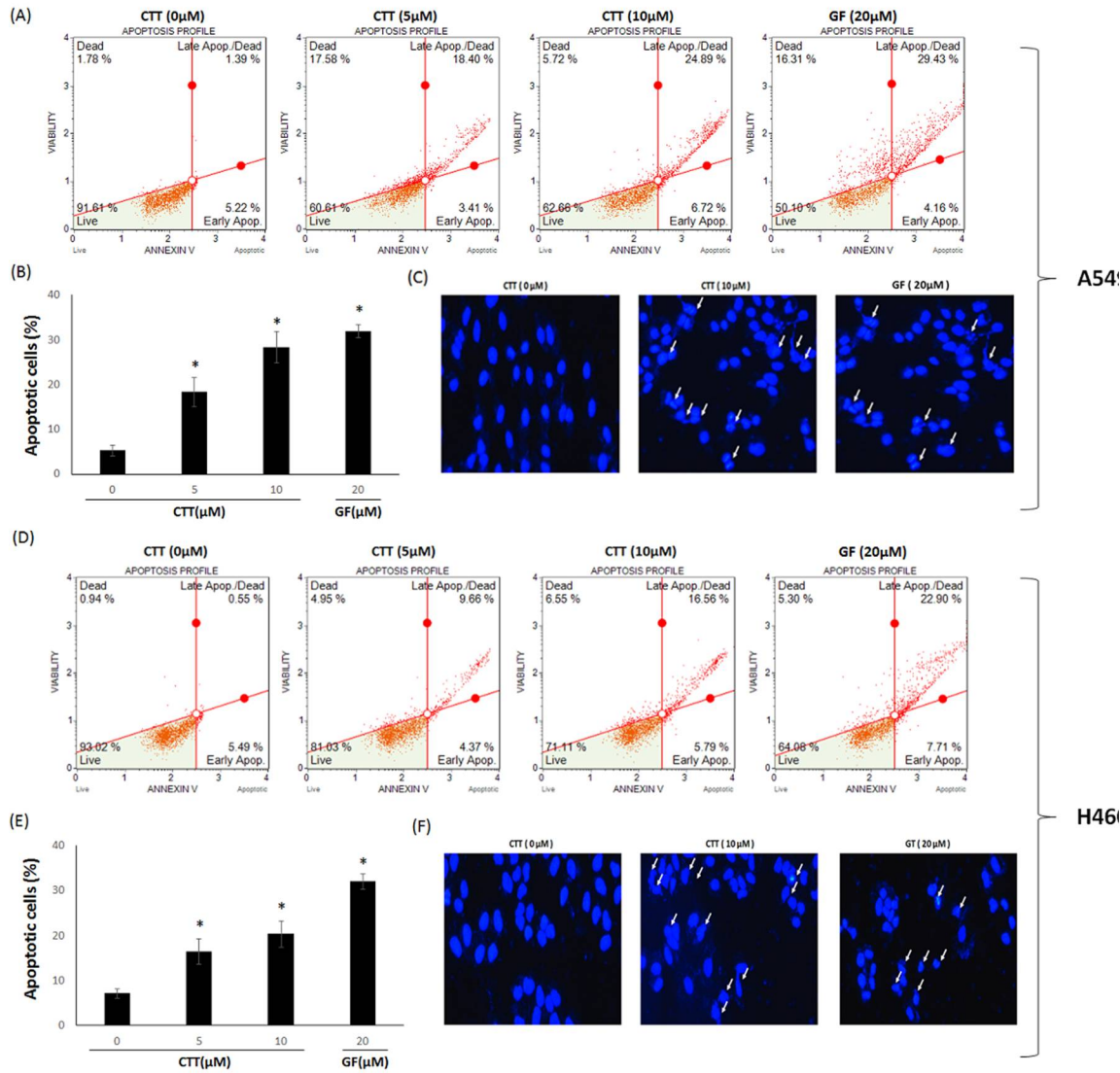


Figure 2. Effects of CTT treatment on apoptosis in A549 and H460 cells. (A) and (D) The cells were treated with 0, 5, or 10 μM of CTT or 20 μM GF (clinical anticancer drug) and stained with Annexin V, PI. After staining, flow cytometry was performed to determine apoptosis. (B) and (E) The histograms of the apoptotic cells were analyzed with MUSE™ Cell Analyzer. (C) and (F) Nuclear condensation and fragmentation after 24 h of treatment with 10 μM CTT or 20 μM GF (clinical anticancer drug), stained with DAPI and visualized by fluorescent microscope (Magnification, 400×). *P < 0.05 compared to the 0 μM of CTT group. The data and images each represent one of the three independent experiments. Significant differences for the treated groups were determined by Duncan's test for multiple comparisons. Values represented as mean ± SD from each experiment.

2.3. CTT affected the expression levels of apoptosis-related proteins in A549 and H460 cells.

To elucidate the mechanism of CTT-mediated apoptosis, apoptosis-related protein expression was measured through western blot analysis. After treatment of CTT in NSCLC cells, the levels of cleaved caspase-3, cleaved caspase-9, cleaved PARP, and Bax were increased. Conversely, the levels of Bcl-2, anti-apoptotic protein, were decreased (Fig. 3A-3D). A549 cells showed more appropriate increase or decrease results than H460 cells in apoptosis-related protein. These results indicate that CTT-induced apoptosis is associated with activating the apoptosis pathway and inhibiting Bcl-2.

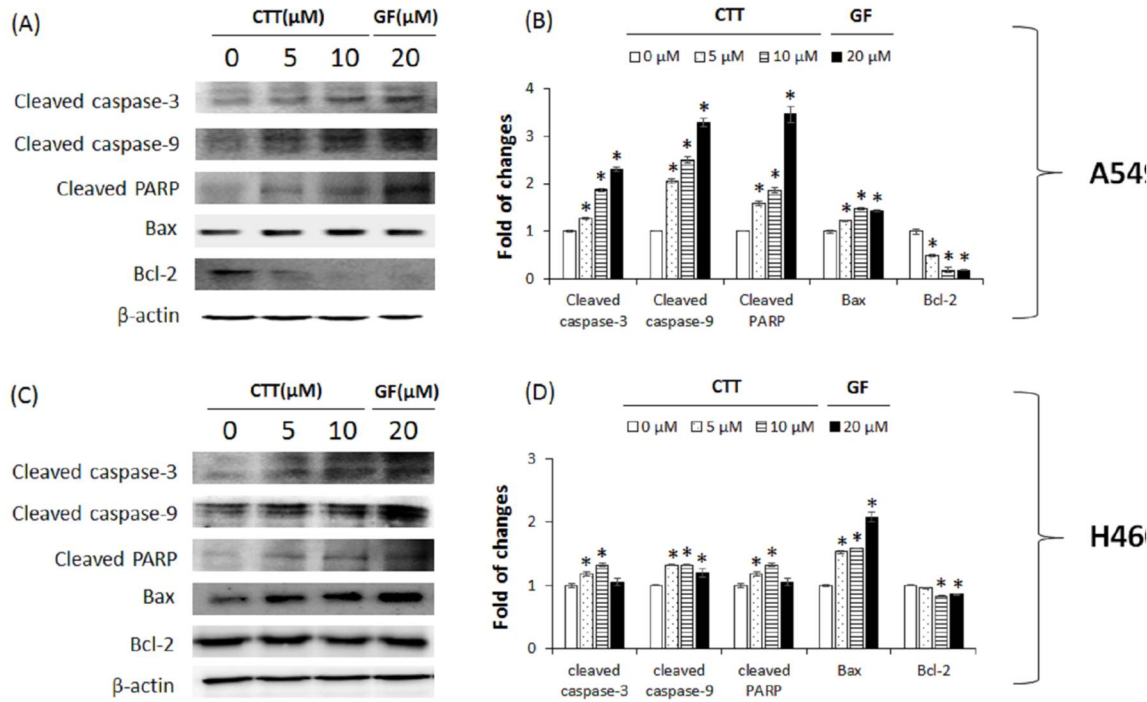


Figure 3. Effects of CTT treatment on the expression of apoptosis-related pathway proteins in A549 and H460 cells. (A) and (C) After treatment with 0, 5, or 10 μM of CTT or 20 μM GF (clinical anticancer drug) for 20 h, the protein levels of cleaved caspase-3, cleaved caspase-9, cleaved PARP, Bax, and Bcl-2 were determined through western blotting. (B) and (D) The calculations of the results were normalized against β-actin. *P < 0.05 compared to the 0 μM of CTT group. The data and images represent each of the three independent experiments. Significant differences for the treated groups were determined by Duncan's test for multiple comparisons. Values represented as mean ± SD from each experiment.

2.4. CTT induced G0/G1 cell cycle arrest in A549 and H460 cells.

To investigate whether the increased apoptosis is related to cell cycle arrest, the number of cells in the G0/G1 phases were analyzed through flow cytometry. The results in CTT-treated A549 (Fig. 4A-B) and H460 (Fig. 4C-D) showed that the percentage of cells in the G0/G1 phases increased significantly next to non-treated cells. These results clearly demonstrate that apoptosis of CTT is related to cell cycle arrest.

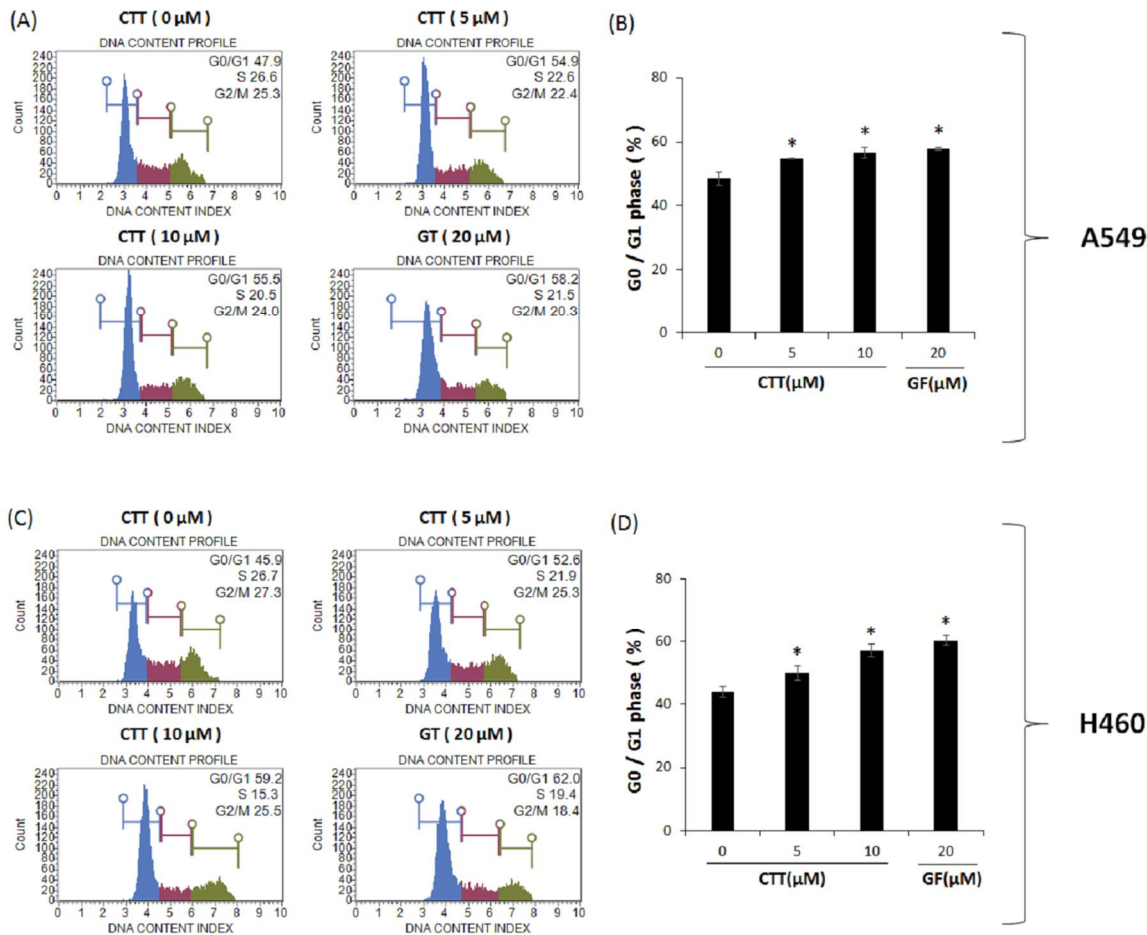


Figure 4. Effects of CTT treatment on G0/G1 phase arrest in A549 and H460 cells. (A) and (C) The cell cycle distribution after 16 h treatment with 0, 5, or 10 μM of CTT or 20 μM GF (clinical anticancer drug) was measured using flow cytometry. (B) and (D) The histogram of the rate of G0/G1 phase cell was analyzed with MUSE™ Cell Analyzer. *P < 0.05 compared to the 0 μM of CTT group. The data represent each of the three independent experiments. Significant differences for the treated groups were determined by Duncan's test for multiple comparisons. Values represented as mean ± SD from each experiment.

2.5. CTT affected the expression levels of proteins related to cell cycle regulatory in A549 and H460 cells.

To verify the mechanism of CTT on G0/G1 arrest, we analyzed the expression levels of proteins involved in the G1 and S phase regulators through Western blot analysis. As shown in Figure 5A-B (A549) and Figure 5C-D (H460), the CTT-treated group showed not only a significant decrease in the expression of the G1 phase (cyclin D, E, and Cdk 4), but also a significant decrease in the expression of the S phase (cyclin A, Cdk 2) compared to the non-treated group. These results reveal that the effects of CTT on G0/G1 phase arrest were induced via changing the expression of proteins related to cell cycle regulation.

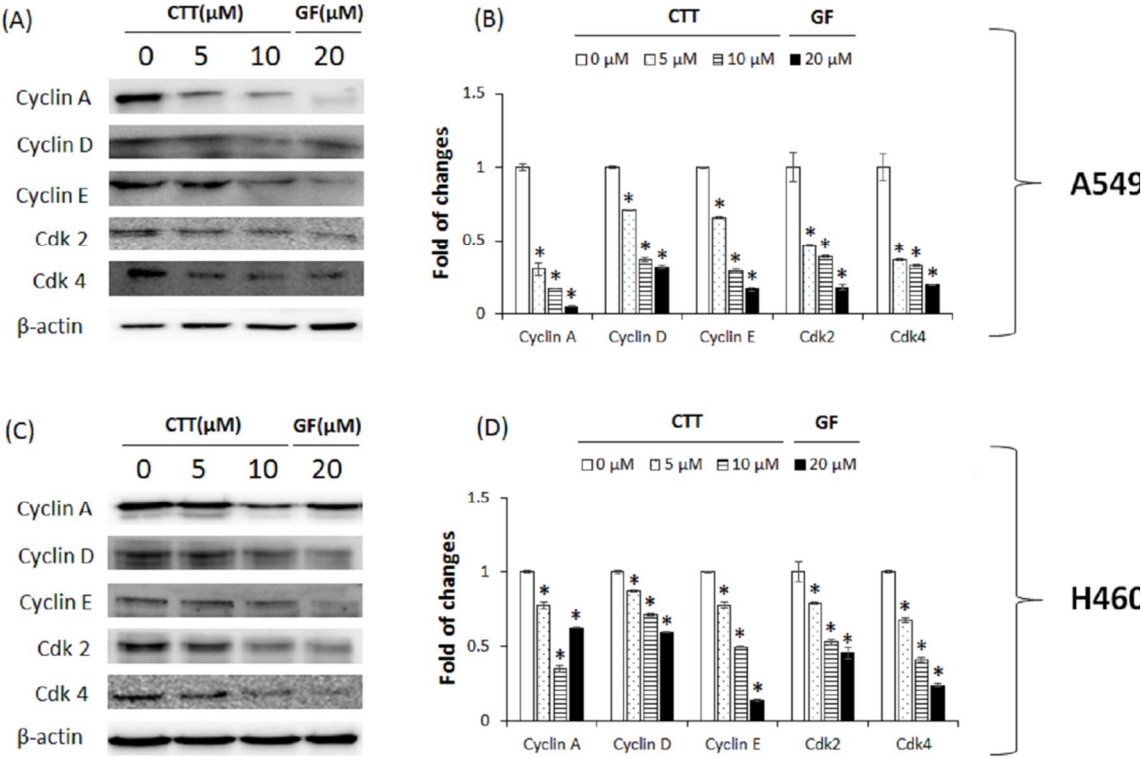


Figure 5. Effects of CTT treatment on the expression of G1 and S cell cycle checkpoint proteins in A549 and H460 cells. (A) and (C) After treatment with 0, 5, or 10 μM of CTT or 20 μM GF (clinical anticancer drug) for 20 h, the protein levels of cyclin A, D, and E and Cdk 2 and 4 were determined through western blotting. (B) and (D) The calculations of the results were normalized against β-actin. *P < 0.05 compared to the 0 μM of CTT group. The data and images represent each of the three independent experiments. Significant differences for the treated groups were determined by Duncan's test for multiple comparisons. Values represented as mean ± SD from each experiment.

2.6. CTT modulated IAP family in A549 and H460 cells.

Western blot analysis was performed in order to further elucidate the members of the IAP family which play an important role in anti-apoptosis in NSCLC cells. After CTT treatment, protein expression levels of the IAP family, such as those of survivin, cIAP-1, and cIAP-2 were significantly decreased (Fig. 6A-D). These results further indicate that the apoptotic effects of CTT in NSCLC were activated by inhibiting not only the Bcl-2 family but also the IAP family.

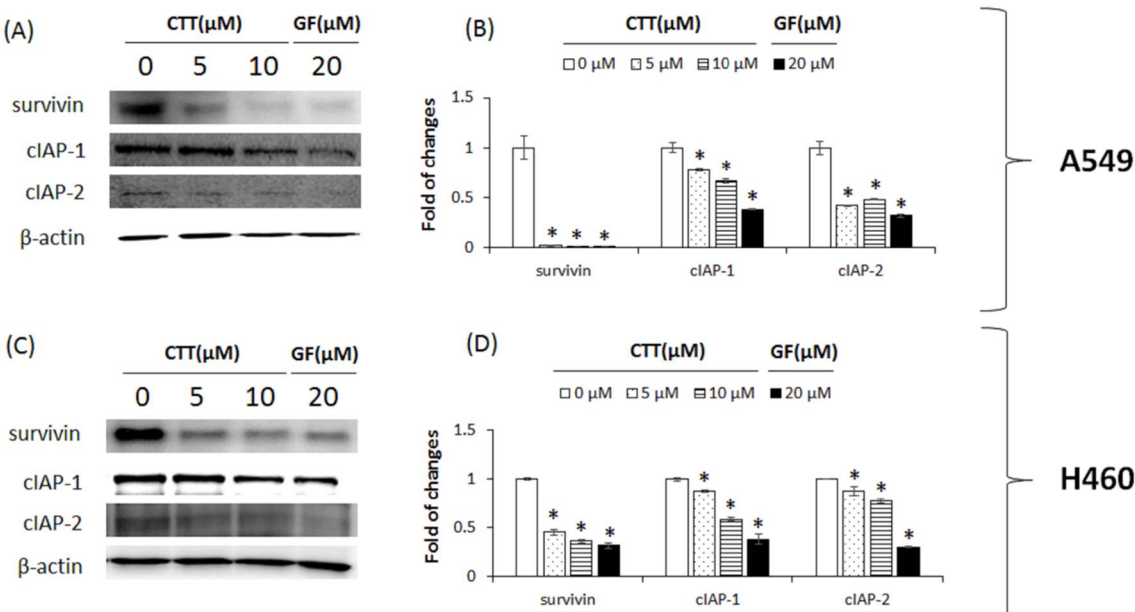


Figure 6. Effects of CTT treatment on the expression of IAP family proteins in A549 and H460 cells. (A) and (C) After treatment with 0, 5, or 10 μM of CTT or 20 μM GF (clinical anticancer drug) for 20 h, the protein levels of cIAP-1, cIAP-2, and survivin were determined through western blotting. (B) and (D) The calculations of the results were normalized against β-actin. *P < 0.05 compared to the 0 μM of CTT group. The data and images represent each of the three independent experiments. Significant differences for the treated groups were determined by Duncan's test for multiple comparisons. Values represented as mean ± SD from each experiment.

2.7. CTT inhibited the PI3K/Akt/GSK-3β pathway.

We performed western blot analysis to examine the PI3K/Akt/GSK3β pathway by measuring the protein expression levels of PI3K, p-Akt/Akt, and p-GSK3β/GSK3β. After CTT treatment, we observed that CTT significantly diminished the expression of PI3K, p-Akt/Akt, p-GSK3β/GSK3β in both cell lines in comparison to the non-treated cells (Fig. 7A-D). These results showed that CTT inhibited the protein expression levels of the PI3K/Akt/GSK-3β pathway.

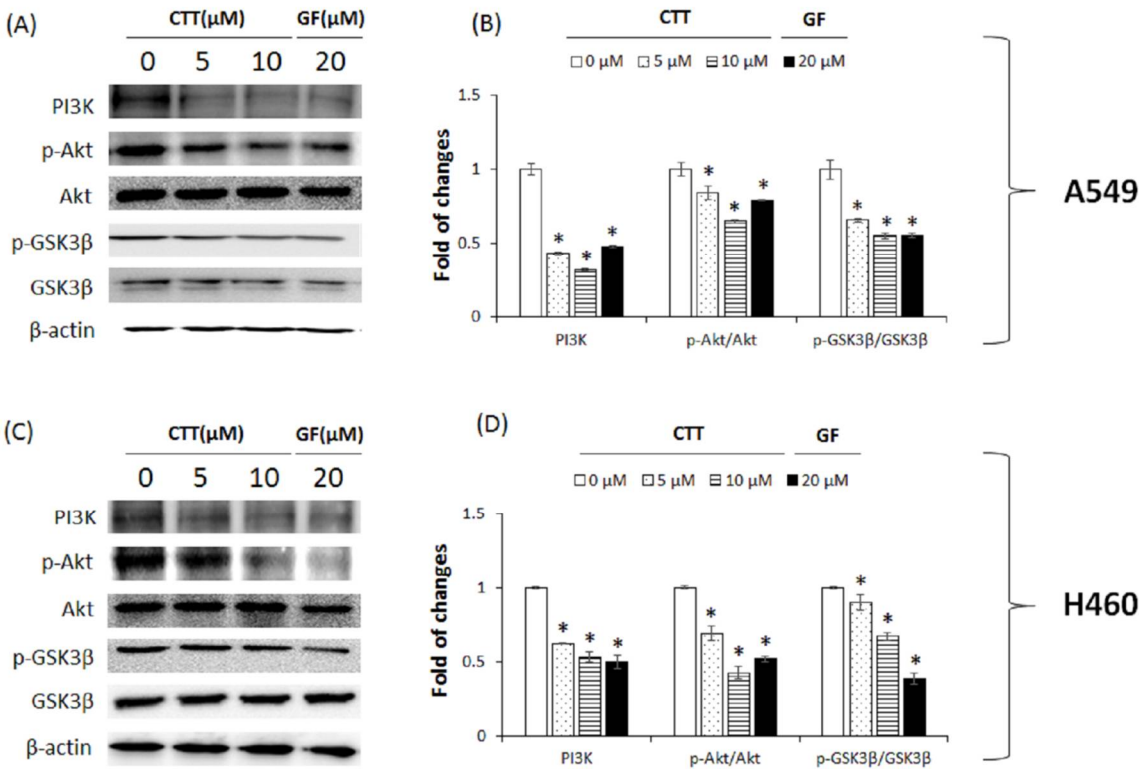


Figure 7. Effects of CTT treatment on the expression of PI3K/Akt/GSK-3β pathway proteins in A549 and H460 cells. (A) and (C) After treatment with 0, 5, or 10 μM of CTT or 20 μM GF (clinical anticancer drug) for 20h, the protein levels of PI3K, p-Akt/Akt, p-GSK3β/GSK3β were determined through western blotting. (B) and (D) The calculations of the results were normalized against specific antibodies or β-actin. Significant differences for the treated groups were determined by Duncan's test for multiple comparisons. Values represented as mean ± SD from each experiment.

3. Discussion

Cell cycle arrest is one way to eliminate cancer cells [15]. In brief, the cell cycle progression is a key process for replicating cells that is accomplished through a series of checkpoints. This checkpoints are activated by DNA damage. In this case, the growth arrest caused by checkpoints allows the cell to repair the damage. If the damage cannot be repaired, the cell is eliminated through apoptosis [16]. According to our experiment results (Figs. 2, 3, and 5), we postulate that CTT has its effect on apoptosis by invigorating the G0/G1 cell cycle arrest. As shown in Fig. 6, we confirmed that checkpoints which are cyclin D/Cdk 4 complex (from G0 into G1 phase), cyclin E/Cdk 2 complex (from G1 – to – S phase transition), and cyclin A (binding with Cdk 2 during S phase) [16] protein levels of CTT-treated cells were significantly diminished compared to those that were non-treated. This indicates that the apoptosis effect of CTT is also related to cell cycle arrest.

Apoptosis is important for maintaining tissue homeostasis. The proper regulation of apoptosis elimination inhibits the general growth of cancer cells [17]. Generally, apoptosis occurs through the extrinsic pathway or intrinsic pathway. These pathways were converged by executioner caspases at the final pathway of apoptosis [18]. Many natural products have been found to induce apoptosis through the intrinsic pathway [19]. The intrinsic pathway is mediated by mitochondria [20]. When DNA damage or oncogene activation occurred, the intrinsic pathway is triggered [21] and the overall intrinsic pathway is regulated by the Bcl-2 family of proteins [22]. The Bcl-2 family proteins which are anti-apoptotic member, Bcl-2, and pro-apoptotic member, Bax are known as key regulators of apoptosis [23].

Cytochrome c, the release of mitochondrial proteins, is mediated by Bax [24]. An increased Bax and decreased Bcl-2 cause a release of cytochrome c from mitochondria to the cytosol where it

activates caspase-9 [25] which subsequently activates the executioner caspase-3 [26]. The executioner caspases quickly begin to cleave such as PARP leading to apoptosis [27].

Inhibitor of apoptosis (IAP) proteins regulate negatively caspases and cell death [28]. IAPs such as survivin, cIAP1 and cIAP-2, are a group of structurally-related proteins that block apoptosis either by binding and inhibiting caspases [29]. As shown Figs. 4 and 7, we observed that when treatment with CTT, pro-apoptotic proteins like cleaved caspase-3, cleaved caspase-9, cleaved PARP, Bax were augmented whereas anti-apoptotic proteins like survivin, cIAP-1, cAP-2 and Bcl-2 were diminished.

The PI3K/Akt cell-signaling pathway is the essential mechanism that is involved in the above-mentioned effects of CTT. Previously published studies have indicated that the PI3K/Akt cell signaling pathway suppresses apoptosis and implements cell proliferation, cycle progression, metastasis of cancer [30-34].

GSK3 β , which is an Akt substrate, is negatively regulated by Akt activity. GSK3 β has two capabilities according to whether it is activated or phosphorylated. Activated GSK3 β is involved in pro-apoptosis but phosphorylated GSK3 β , because of its Akt activity, interrupt the apoptotic activity [35,36]. As presented in Fig. 8, our results showed markedly diminished protein expression in PI3K, p-Akt/Akt, and p-GSK-3 β /GSK-3 β signaling pathway factors.

Altogether, this study certifies the anticancer effect of CTT in NSCLC by inducing G0/G1 cell cycle arrest and apoptosis through PI3K/Akt/GSK3 β signaling pathway inhibition. So, CTT could be a potential therapeutic natural agent for targeting the PI3K/Akt/GSK3 β in NSCLC.

4. Materials and Methods

4.1. Reagent.

Fetal bovine serum (FBS), penicillin/streptomycin were purchased from Hyclone (Logan, UT, USA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), Bovine serum albumin (BSA), Dimethyl sulfoxide (DMSO), 4',6-diamidino-2-phenylindole (DAPI), and Gefitinib (GF) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for cleaved caspase-3 (#9661), cleaved caspase-9 (#9505), cleaved PARP (#9541), survivin (#2808), Cdk4 (#12790), Cyclin D1 (#2978), Phospho-Akt (#4058), and Akt (#9272) were purchased from Cell Signaling (Cell Signaling Technology, Beverly, MA). Cdk2 (sc-6248), Cyclin A (sc-751), Cyclin E (sc-198), Bcl-2 (sc-7382), Bax (sc-7480), PI3K (sc-602), Phospho-GSK-3 β (sc-81494), GSK-3 β (sc-81462), cIAP-1 (sc-271419), cIAP-2 (sc-517317), β -actin (sc-47778), and Peroxidase-conjugated secondary antibodies rabbit (sc-2357) and mouse (sc-516102) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

4.2. Preparation of CTT.

Cryptotanshinone (CTT) (C5624, $\geq 98\%$ (HPLC)) (Fig.8) was purchased from Sigma-Aldrich (St. Louis, MO). Each concentration of CTT (0, 5, and 10 μ M) and 20 μ M GF was dissolved with DMSO.

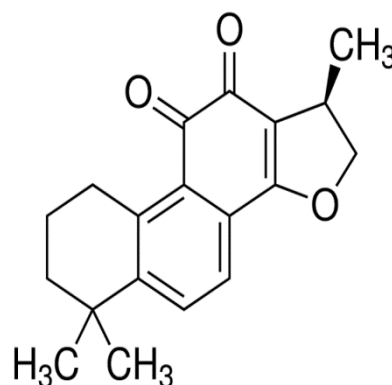


Figure 8. Structure of Cryptotanshinone (CTT).

4.3. Cell culture.

The human NSCLC cell line A549 and H460 were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and grown in RPMI 1640 (Thermo Fischer Scientific, MA, USA) containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO₂ in air. CTT was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mM and stored at 4°C. The final concentration of DMSO in all experiments did not exceed 0.1% and the final CTT solutions were all diluted in the fresh culture medium.

4.4. Cell viability.

The effects of CTT on A549 and H460 cell viability were determined through an MTS assay. Cells were seeded in 96-well culture plates (2×10³ cells/well) and incubated for 24 h. GF was used a positive control. Following incubation, various concentrations (0, 5, or 10 µM) of CTT or 20 µM GF were added and incubated for 24, 48, and 72 h. The medium was replaced with MTS solution and each culture well was optimized with a Micro plate reader (Titertek Multiskan, Flow Laboratories, North Ryde, Australia) at 490 nm. Cell viability was calculated using the following formula (1):

$$\text{Cell viability (\%)} = (\text{OD 490 value of CTT treated cells} / \text{OD 490 value of untreated cells}) \times 100 \quad (1)$$

4.5. LDH assay.

The effects of CTT on A549 and H460 LDH release were detected using the Pierce® LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions. In brief, the cells were seeded in 96-well plates (2×10³ cells/well) for 24 h, then treated with CTT (0, 5, or 10 µM) or GF (20 µM) for 24 h. Next, the maximum LDH well was added to a 10 µl 10 X lysis buffer. After 45 min, the supernatants were transferred into a new 96-well plate, and 50 µl reaction mixture was added to each well. Finally, the supernatants were incubated for 30 min in room temperature and 50 µl stop solution were put into each well. LDH release was measured at 490 nm and 680 nm of absorbance and calculated using the following formula (2):

$$\text{LDH release (\%)} = [(\text{OD 490-OD 680 value of CTT treated cells}) - (\text{OD 490-OD 680 value of untreated cells})] / (\text{OD 490-OD 680 value of maximum LDH cells} - \text{OD 490-OD 680 value of untreated cells}) \times 100. \quad (2)$$

4.6. DAPI staining.

Cell nuclear morphology was measured through fluorescence microscopy following DAPI staining. A549 and H460 cells were treated with CTT for 24h. Cells were then washed with PBS, fixed with 4 % formaldehyde and washed 2 times each for 5min. Next, cells were stained with DAPI, and incubated for 5 min at room temperature away from light. The cells were finally washed with PBS one time and visualized using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

4.7. Cell cycle analysis.

Cell cycle components were analyzed with Muse™ Cell cycle reagent (EMD Millipore Corp. Billerica, MA, USA). A549 and H460 cells (10×10⁴ cells/well) were seeded in 6-well plates and treated with CTT (0, 5, or 10 µM) or GF 20 µM for 16 h. After being harvested from the culture medium, cells were washed with 1 mL PBS, then fixed with ice-cold fresh 70% EtOH for over 3 h. Next, cells were washed with PBS and had Muse™ Cell cycle reagent added to them. Then, cells were incubated at room temperature for 30 min without light. Cells were analyzed with Muse cell analyzer and Muse analysis software (Merck Millipore).

4.8. Annexin V assay.

The extent of apoptosis was determined by Muse Annexin V and a dead cell kit (Millipore, Billerica, MA, USA). According to the manufacturer's procedure, A549 and H460 cells (10×10⁴ cells/well) were seeded in 6-well plates and treated with CTT (0, 5, or 10 μM) or GF 20 μM for 24 h. After being harvested from the culture medium, cells were washed with 1mL PBS and had 100 μL of Muse™ Annexin V Dead Cell reagent added to them. Next, cells were incubated at room temperature for 20 min without light. The apoptosis was then analyzed with Muse cell analyzer and Muse analysis software (EMD Millipore).

4.9. Western blotting.

After treatment of A549 and H460 cells with CTT (0, 5, or 10 μM) or GF 20 μM for 20 h, harvested cells were lysed with a lysis buffer (iNtRon Biotech, Seoul, Republic of Korea) for 20 min. The cell lysates were centrifuged for five min at 13,000 rpm and protein concentration was determined through bicinchoninic acid (BCA) assay. The cell lysates were mixed with a 3 X sample buffer, inserted to SDS gel electrophoresis, and then transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5 % skim milk or 3 % BSA for 1 h and probed overnight at 4°C with primary antibodies. After a series of washes, the membrane was incubated for 1 h at room temperature with a secondary antibody conjugated with horseradish peroxidase (HRP). Next, the proteins were supplemented with ECL prime Western blotting detection reagents (GE Healthcare, UK). ImageQuant LAS 4000 Mini Biomolecular Imager (GE Healthcare, UK) was used for analyzing the Bands.

4.10. Statistical analyses.

The statistical analysis was performed using one-way analysis of variance, followed by Duncan's test for various comparisons. Results are presented as the means ± standard deviations of the independent experiments. All calculations were performed using SPSS statistics 23 software (SPSS Inc. Chicago, IL, USA). Comparison with p < 0.05 were considered to be statistically significant.

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Conflicts of Interest: The authors declare no conflict of interest.

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