Signal transducer and activators of transcription 3 regulates cryptotanshinone-induced apoptosis in human mucoepidermoid carcinoma cells

Hyun-Ju Yu*, Chul Park[1,*], Sun-Ju Kim[2], Nam-Pyo Cho, Sung-Dae Cho

Departments of Oral Pathology and Cancer Biology, School of Dentistry, and Institute of Oral Bioscience, [1]Department of Veterinary Internal Medicine, College of Veterinary Medicine, Chonbuk National Univerisity, Jeonju, 561-756, [2]Department of Dental Hygiene, Division of Health Sciences, Cheongju University, Cheongju, 360-764, South Korea

Submitted: 23-01-2014 Revised: 18-04-2014 Published: 30-08-2014

ABSTRACT

Background: Cryptotanshinone (CT) is a biologically active compound from the root of *Salvia miltiorrhiza* that has been reported to induce apoptosis in various cancer cell lines; but, it has not yet been fully explored in human mucoepidermoid carcinoma (MEC). **Objective:** Here, we demonstrated the apoptotic effects and its related mechanism in MC-3 and YD-15 human MEC cell lines. **Materials and Methods:** The effects of CT on apoptotic activity were evaluated by cell proliferation assay, Western blotting, 4′-6-diamidino-2-phenylindole staining, reverse transcription-polymerase chain reaction, and luciferase assay. **Results:** Our data show that CT treatment of MC-3 cells results in anti-proliferative and apoptotic activities in MC-3 and it is accompanied by a decrease in phosphorylation and dimerization of signal transducer and activators of transcription 3 (STAT3). CT decreased the expression levels of myeloid cell leukemia-1 (Mcl-1) and surviving, whereas Bcl-xL expression was not changed. CT clearly regulates survivin protein at a transcriptional level and alters Mcl-1 through proteasome-dependent protein degradation. In addition, CT-induced apoptotic cell death in YD-15, another human MEC cell line, was associated with the inhibition of STAT3 phosphorylation. **Conclusion:** These data suggest that CT could be a good apoptotic inducer through modification of STAT3 signaling in human MEC cell lines.

Key words: Apoptosis, cryptotanshinone, myeloid cell leukemia-1, mucoepidermoid carcinoma, signal transducer and activator of transcription 3, survivin

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.139802

Quick Response Code:

INTRODUCTION

Signal transducer and activator of transcription 3 (STAT3) belongs to the STAT family of proteins, which are latent cytoplasmic transcription factors that convey signals from the cell surface to the nucleus through cytokines and growth factors.[1] STAT3 has an important role in basic processes including apoptosis, development, differentiation, and proliferation.[2] The activation of STAT3 signaling is initiated by phosphorylation and phosphorylated STAT3 then dimerizes to translocate into the nucleus during normal biological conditions. During maintenance of cell homeostasis, STAT3 activation is rapid and transient. However, constitutive activation of STAT3 is associated with many human cancers.[3,4] Previously, Grandis *et al.*[5] have reported that oral cancer also exhibits constitutive activation of STAT3 signaling suggesting that STAT3 signaling can be a good molecule for oral cancer including mucoepidermoid carcinoma (MEC).

Cryptotanshinone (CT), one of the major tanshinones isolated from *Salvia miltiorrhiza* Bunge (Danshen) has been used in Chinese medicine for the treatment of various diseases including coronary artery disease, hyperlipidemia, acute ischemic stroke, and Alzheimer's disease.[6-8] CT also has the unique biological activity of inhibiting the phosphorylation of STAT3, and thus it has been categorized as a STAT3 inhibitor. Recently, several groups reported that CT arrests cell cycle and induces apoptosis in several cancer cell lines.[9-11] However, there have not been any reports on the possible anticancer activities of CT in human MEC cell lines.

In this study, we investigated the apoptotic effects of CT and the mechanism by which it regulates STAT3 in two human MEC cell lines (MC-3 and YD-15). Our results

Address for correspondence:
Prof. Sung-Dae Cho, Departments of Oral Pathology and Cancer Biology, School of Dentistry, and Institute of Oral Bioscience, Chonbuk National University, Jeonju, 561-756, Republic of Korea.
E-mail: efiwdsc@chonbuk.ac.kr

* These authors contributed equally to this work

provide that CT can inhibit STAT3 signaling in order to exert apoptotic activity through blocking phosphorylation and dimerization of STAT3.

MATERIALS AND METHODS

Chemicals and antibodies

Cryptotanshinone [Figure 1a], 4'-6-diamidino-2-phenylindole (DAPI) and cycloheximide (CHX) were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). The actin antibody and MG-132 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies for cleaved poly ADP ribose polymerase (PARP), cleaved caspase-3, p-STAT3, STAT3, survivin, myeloid cell leukemia-1 (Mcl-1) and Bcl-xL were purchased from Cell Signaling Technology Inc. (Charlottesville, VA, USA).

Cell culture and chemical treatment

MC-3 cells were kindly provided by Dr. Wu Junzheng from Forth Military Medical University (Xi'an, China) and YD-15 cells were obtained from Yonsei University (Seoul, Korea). MC-3 cells were grown in DMEM, and YD-15 cells were grown in RPMI-1640; both types of media were supplemented with 10% fetal bovine serum in CO_2 incubator. An equal number of cells were seeded. When cells reached at 50-60% confluence, they were treated with DMSO or various concentrations of CT.

Cell viability assay

The cell lines were treated with different concentrations of CT (2, 4, or 8 μM for MC-3; 5, 10, or 15 μM for YD-15) for 24 h. The number of surviving cells was counted using a hemacytometer with 0.4% of trypan blue. Each experiment was performed in triplicate, and the results were expressed as the percentage of surviving cells compared to DMSO treatment group.

Western blotting

Cell lysates were extracted with lysis buffer and quantified with a DC Protein Assay kit (Bio-RAD, Hercules, CA, USA). Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Bio-RAD). Membranes were blocked with 5% skim milk in TBST buffer at room temperature (RT) for 1 ~ 1.5 h, washed with TBST, and maintained overnight at 4°C with designated primary antibodies. Subsequently, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody at RT for 2 h. Antibody-bound proteins were detected using an ECL Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc.).

4'-6-diamidino-2-phenylindole staining

Evaluation of fragmentation and condensation in the nuclei of apoptotic cells was performed using DAPI. After CT treatment, cells were harvested by trypsinization and fixed in 100% methanol for 10 min at RT. The cells were resuspended in phosphate-buffered saline, deposited on poly-L-lysin coated slides, and stained with a DAPI solution. Nuclear condensation

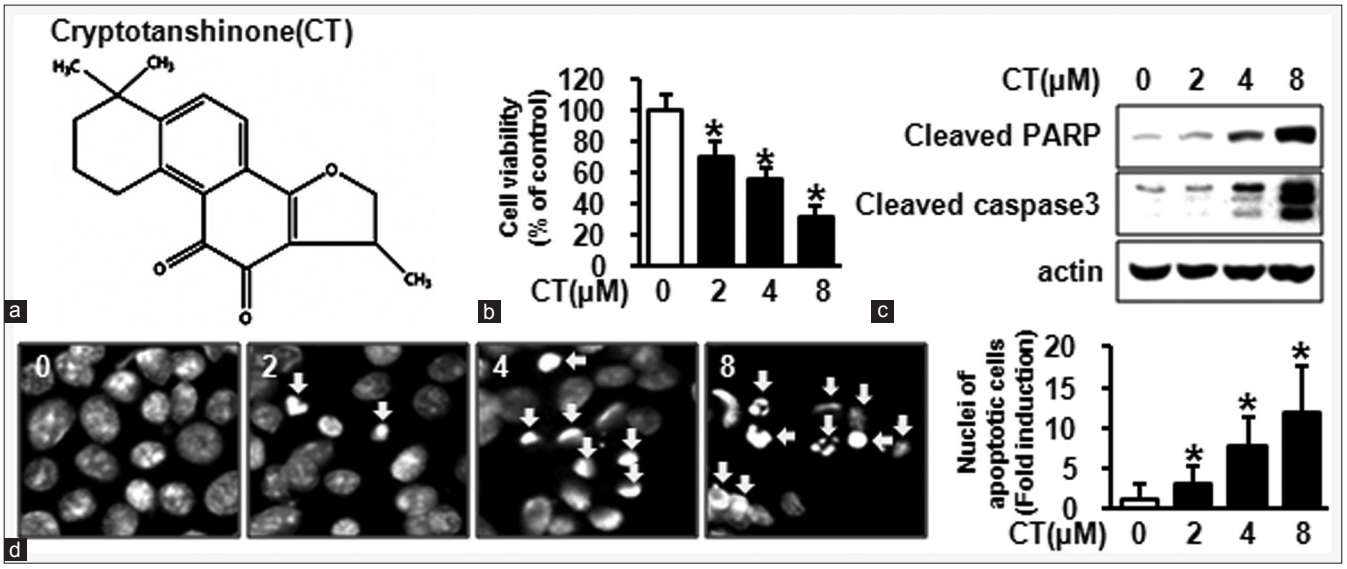

Figure 1: The effect of cryptotanshinone (CT) on cell viability and apoptosis in MC-3 cells. (a) Chemical structure of CT. MC-3 cells were treated with 2, 4, or 8 μM of CT for 24 h. Cell viability was determined using a trypan blue exclusion assay. (b) The apoptotic effect of CT was determined by Western blot analysis using the antibodies against cleaved poly ADP ribose polymerase and cleaved caspase 3 (c) and 4'-6-diamidino-2-phenylindole staining. (d) Results are expressed as means ± standard deviation for triplicate experiments and significance ($P < 0.05$) compared with dimethyl sulfoxide-treated cells is given (*)

and fragmentation were detected under a fluorescence microscope.

Cross-linking

For STAT3 dimerization, the cells were suspended in conjugation buffer with 10 mM EDTA. The cell lysates were incubated with 0.2 mM bismaleimide (Thermo scientific, Rockford, IL, USA) at RT for 1 h, and then extracted with lysis buffer for Western blotting.

Reverse transcription-polymerase chain reaction

After chemical treatment, RNA was isolated using an easy-blue total RNA Extraction kit (iNtRON, Seongnam, Korea). RNA was used to synthesize cDNA using a RT System (Promega, Madison, WI, USA). The following primer sequences were used; survivin: 5'-ATG GCC GAG GCT GGC TTC ATC-3' (S), 5'-ACG GCG CAC TTT CTT CGC AGT T-3' (AS); Mcl-1 5'-TGC TGG AGT TGG TCG GGG AA-3' (S), 5'-TCG TAA GGT CTC CAG CGC CT-3' (AS); β-actin 5'-GTG GGG CGC CCC AGG CAC CA-3'(S), 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'(AS). Polymerase chain reaction (PCR) conditions were followed: 28 cycles: 60 s at 95°C, 60 s at 60°C and 90 s at 72°C for survivin and Mcl-1, and 25 cycles: 60 s at 95°C, 60 s at 60°C and 90 s at 72°C for β-actin. PCR products were analyzed by 1-2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Dual-luciferase assay

Promoter activity was evaluated using a Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's instructions. In brief, cells were seeded in 12-well plates and transfected with a human survivin and Mcl-1 luciferase reporter plasmid using Lipofectamine 2000 reagent (Invitrogen) and cultured for 6 h. The culture medium was removed, and cells were treated with CT for 24 h. Cells were lysed by incubation for 15 min with 1× passive lysis buffer and cell debris was removed by centrifugation. The supernatant was assayed for Firefly and Renilla luciferase activities using a Microbeta Trilux 1450 luminescence counter (Perkin Elmer, Turku, Finland).

Statistical analysis

Data were assessed for statistical significance using Student's *t*-test. A value of $P < 0.05$ compared to the vehicle control was considered to be statistically significant.

RESULTS

Cryptotanshinone decreases cell viability and induces apoptosis in human mucoepidermoid carcinoma MC-3 cells

To investigate the inhibitory effect of CT on cell growth, MC-3 cells were treated with DMSO or different concentrations of CT (2, 4, and 8 μM) for 24 h. As shown in Figure 1b, treatment with CT for 24 h significantly decreased the viability of MC-3 cells in a concentration-dependent manner. To understand how CT decreases cell viability, the effect of CT on apoptosis was assessed using Western blotting and DAPI staining. We found that CT caused increases in the cleavages of PARP and caspase-3 [Figure 1c]. The distinct features of apoptotic cells, nuclear condensation and fragmentation were also significantly increased in the CT-treated group compared with the DMSO-treated group [Figure 1d]. These results suggest that the inhibition of cell viability by CT is attributed to the induction of apoptotic cell death.

Cryptotanshinone regulates survivin and myeloid cell leukemia-1 expression through the inactivation of signal transducer and activators of transcription 3 kinase

Because CT was previously identified as an inhibitor of STAT3,[12] we hypothesized that CT may inactivate STAT3 signaling in MC-3 cells. To test this hypothesis, the effect of CT on STAT3 phosphorylation in MC-3 cells was investigated. The results showed that CT significantly reduced STAT3 phosphorylation [Figure 2a]. STAT3 phosphorylation causes its homo-dimerization and subsequent translocation to the nucleus for binding to specific DNA sequences in the promoters of target genes.[4,13] Therefore, we analyzed the effect of CT on STAT3 dimerization. As shown in Figure 2b, CT clearly decreased the amounts of dimerized STAT3 concentration-dependently. Dimerized STAT3 can regulate the expression of target proteins involved in cell proliferation, survival, and angiogenesis.[14,15] We investigated whether the expression levels of survivin, Mcl-1 and Bcl-xL were affected by CT and the results showed that CT suppressed the expression of survivin and Mcl-1 protein, but did not affect Bcl-xL protein levels [Figure 2c]. These results suggest that CT has an effect on STAT3 signaling that results in a decrease in survivin and Mcl-1 protein.

Cryptotanshinone decreases survivin protein at the transcriptional level and alters myeloid cell leukemia-1 protein expression by the posttranslational modification

To determine how CT regulates survivin and Mcl-1 proteins, we examined the expression levels of survivin and Mcl-1 mRNA and their promoter activities using RT-PCR and dual-luciferase assay in MC-3 cells. The results showed that CT significantly attenuated survivin mRNA and its promoter activity, but Mcl-1 mRNA levels and promoter activity were not altered in MC-3 cells [Figure 3a and b]. The effect of CT on Mcl-1 protein

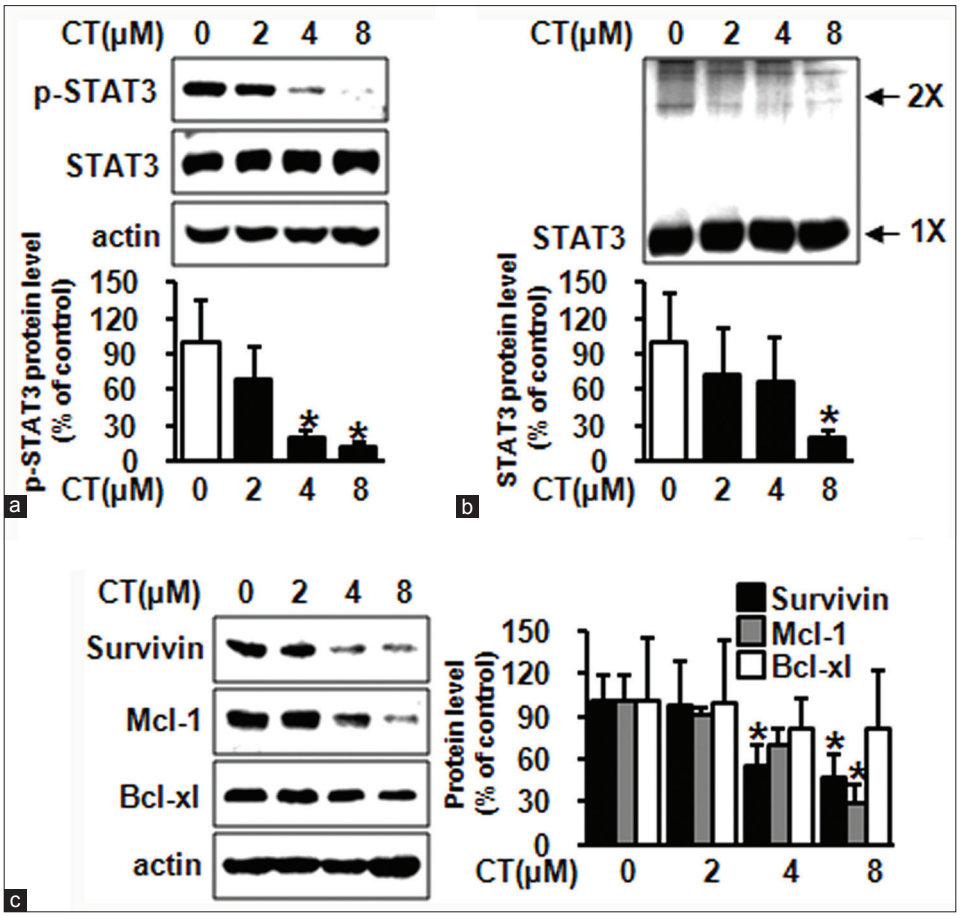

Figure 2: The effect of cryptotanshinone (CT) on signal transducer and activators of transcription 3 (STAT3) signaling. MC-3 cells were treated with 2, 4, or 8 μM of CT for 24 h. p-STAT3 and STAT3 were analyzed by Western blot analysis. (a) STAT3 dimerization was performed by Western blot analysis after treatment with cross-linker BMH. (b) Survivin, myeloid cell leukemia-1 and Bcl-xL proteins were determined in MC-3 cells treated with CT by Western blot analysis. (c) All data are expressed as means ± standard deviation for triplicate experiments and significance ($P < 0.05$) compared with dimethyl sulfoxide-treated cells is indicated (*)

turnover after the blockage of new protein synthesis by CHX was examined in order to elucidate a potential mechanism for the suppression of Mcl-1 protein by CT in MC-3 cells. Our results showed that co-treatment with CT and CHX significantly decreased Mcl-1 protein levels [Figure 3c]. To investigate the involvement of proteasomes in CT-mediated protein degradation, we used MG-132, a proteasome inhibitor. The results showed that the down-regulation of Mcl-1 protein by CT was reverted in the presence of MG-132 suggesting that CT significantly accelerated Mcl-1 proteasome-dependent protein degradation [Figure 3d]. We also checked phospho-eIF4E (cap-dependent translation initiation factor) to investigate its effect on the process of Mcl-1 protein synthesis and the result showed that CT clearly increased its expression level (data not shown). These findings indicate that CT affects the expression of survivin and Mcl-1 protein through transcriptional modification and proteasome-dependent protein degradation, respectively.

Inhibition of signal transducer and activators of transcription 3 phosphorylation by cryptotanshione-induces apoptosis in YD-15 cells

To evaluate whether the modification of STAT3 signaling by CT generally occurred in human MEC cell line during apoptosis, YD-15 cell, another human MEC cell line was used. Figure 4a showed that CT effectively inhibited the viability of YD-15 cells. CT clearly induced caspase-related PARP cleavage [Figure 4b] and increased the number of YD-15 cells displaying nuclear condensation and fragmentation [Figure 4c]. We also found that CT significantly inhibited STAT3 phosphorylation [Figure 4d]. These data suggest that CT also decreases STAT3 phosphorylation in order to induce apoptosis in another MEC cell line. Figure 5 is the summary of the molecular mechanism by which the treatment with CT inhibits the STAT3 signaling pathway to induce apoptosis in MEC cell lines through regulation of survivin and Mcl-1 expression.

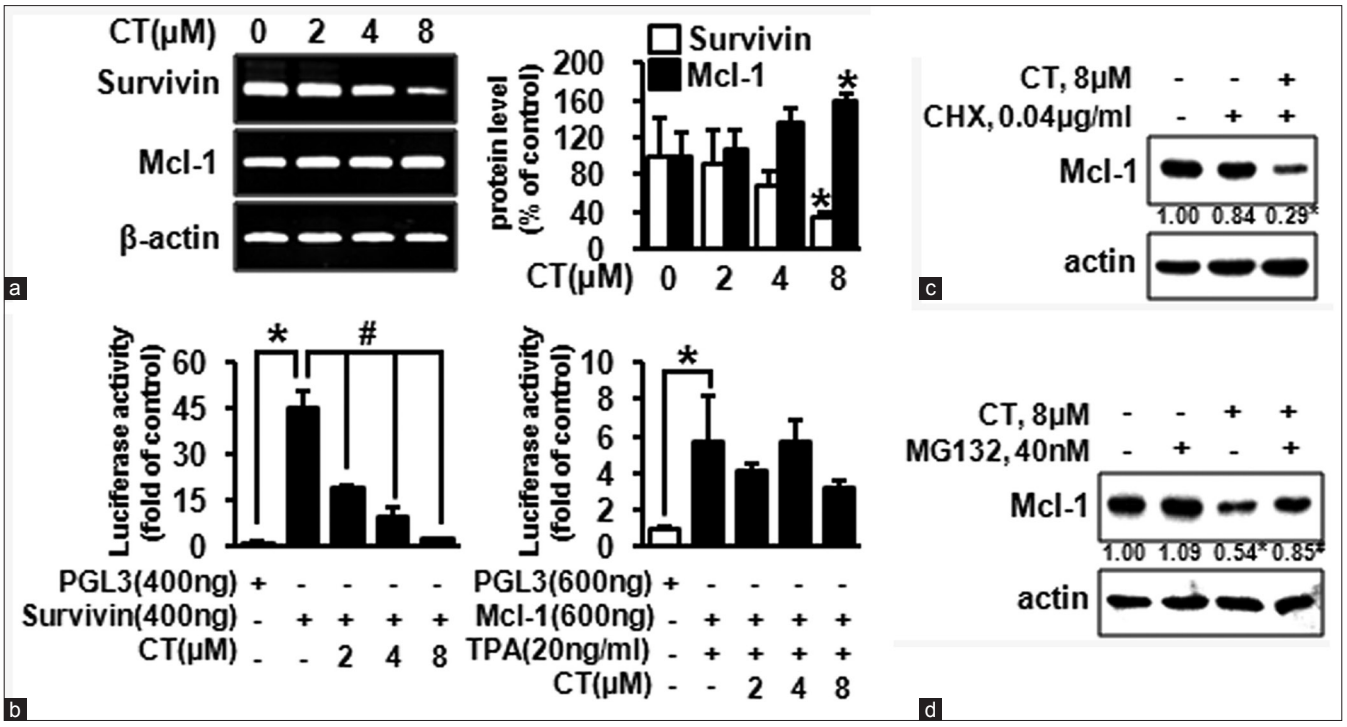

Figure 3: The effects of cryptotanshione (CT) on survivin and myeloid cell leukemia-1 (Mcl-1). Survivin and Mcl-1 mRNA levels were confirmed by RT-PCR in MC-3 cells and normalized to β-actin. (a) MC-3 cells were transfected with PGL3 vector control (white bar) or survivin and Mcl-1 promoter construct (black bar), treated with CT, and induction of luciferase activity was determined. (b) The effect of CT on Mcl-1 protein turnover was determined by Western blot analysis in MC-3 cells treated with the protein synthesis inhibitor, cycloheximide (0.4 μg/ml) with/without CT (8 μM) for 24 h. (c) MC-3 cells were pretreated for 1 h with the proteasome inhibitor, MG-132 (40 nM) or dimethyl sulfoxide (DMSO) and CT (8 μM) or DMSO were added for 3 h. Then, Mcl-1 expression was determined by Western blot analysis. (d) All data are expressed as means ± standard deviation for triplicate experiments and significance ($P < 0.05$) compared with DMSO-treated cells is indicated (*)

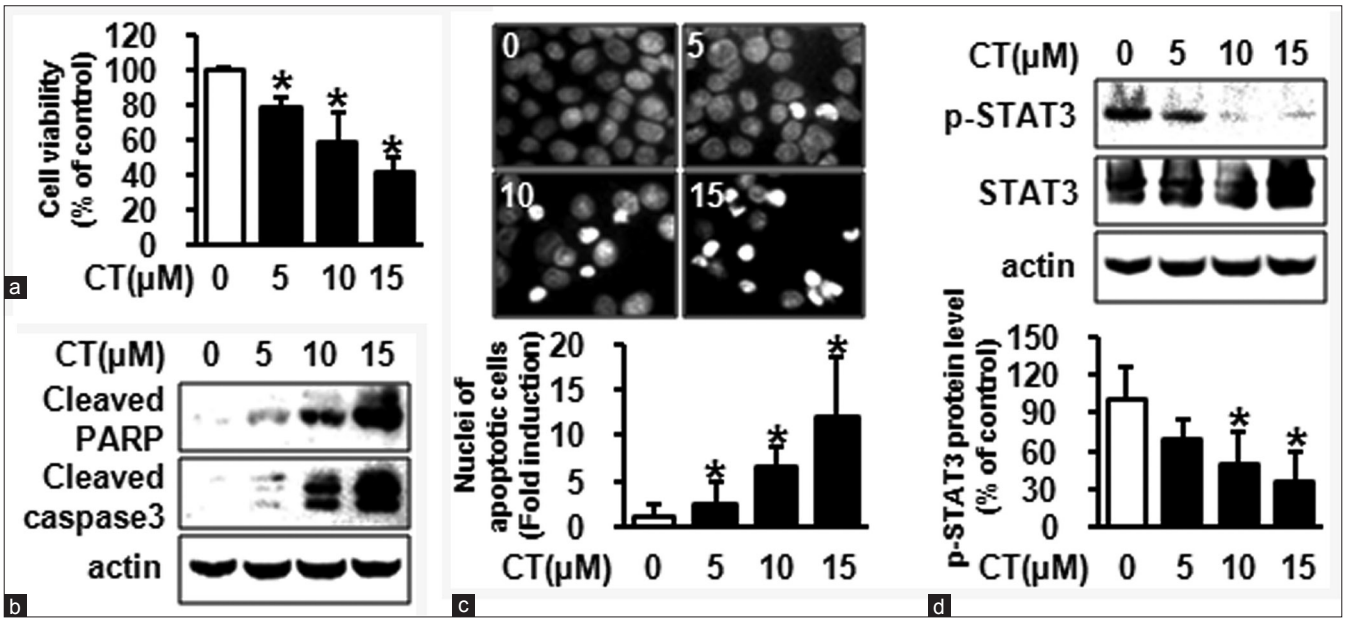

Figure 4: The effect of cryptotanshione (CT) on apoptosis and signal transducer and activators of transcription 3 (STAT3) in YD-15 cells. YD-15 cells were treated with 5, 10, or 15 μM of CT for 24 h. Cell proliferation was determined using a trypan blue exclusion assay. (a) The apoptotic effect of CT was determined by Western blot analysis using the antibodies against cleaved poly ADP ribose polymerase and cleaved caspase 3 (b) and 4'-6-diamidino-2-phenylindole staining. (c) p-STAT3 and STAT3 were analyzed by Western blot analysis. (d) Results are expressed as means ± standard deviation for triplicate experiments and significance ($P < 0.05$) compared with dimethyl sulfoxide-treated cells is indicated (*)

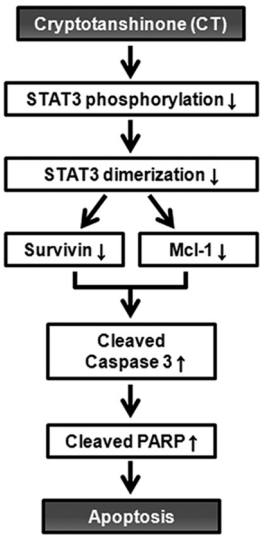

Figure 5: A model for the molecular mechanism by which the treatment with cryptotanshinone decreases the signal transducer and activators of transcription 3 signaling pathway to induce apoptosis in mucoepidermoid carcinoma cell lines through regulation of survivin and myeloid cell leukemia-1

DISCUSSION

The purpose of this study was to demonstrate whether CT could suppress the proliferation of MEC cells by interfering with STAT3 pathways. We have shown that CT has significant growth-inhibitory and apoptotic effect in MC-3 cells. In agreement with our present study, other group also reported that CT treatment induced programmed cell death in various cancer cell lines.[11,16,17] These results suggest that CT can be a good inducer of apoptosis in cancer cells.

The STAT protein family includes seven proteins that mediate the transduction of extracellular signals for the transcription of target genes. Among the STATs, STAT3 is closely associated with tumorigenesis.[18,19] Constitutive activation of STAT3 represents one of the critical events in multistep carcinogenesis.[20] Recently, it was found that the inhibition of STAT3 induces apoptosis in human prostate cancer cell lines[21,22] implying that STAT3 can be useful for the treatment of various cancer as a potential molecular target. Previously, it was reported that CT is one of the major active components of Danshen as a small-molecule inhibitor of STAT3 transcriptional regulatory activity.[6] In this study, we confirmed that CT treatment inhibited STAT3 phosphorylation, which may be related to CT-induced apoptosis in MEC cell lines. STAT3 activation was initiated upon phosphorylation and involved dimerization between two phosphorylated STAT3 monomers.[3,14,23] Thus, we investigated the effect of CT on STAT3 dimerization and found that CT clearly reduced STAT3 dimerization, which is consistent with the findings from a previous report.[12]

Signal transducer and activators of transcription 3 phosphorylation and homo-dimerization cause subsequent translocation of STAT3 to the nucleus where it affects the expression of target proteins.[4,13] Survivin is highly expressed in various types of human cancers, including breast, colon, liver, lung, ovarian, pancreas, prostate, stomach, and hematopoietic malignancies, as well as melanoma.[24] Our previous studies also showed that survivin is a key mediator for MEC cell lines and several compounds blocked survivin during apoptotic cell death.[25,26] It was recently shown that constitutive activation of STAT3 can modify the expression level of survivin protein in malignant cells[27,28] suggesting that survivin may be regulated by the STAT3 signaling pathway. Thus, we investigated the effects of CT on survivin and the results showed that CT clearly reduced the expression level of survivin protein at a transcriptional level evidenced by the suppression of mRNA and its promoter activity in response to CT. Several pieces of evidence demonstrate that the abnormal activity of STAT3 promotes tumorigenesis by increasing the expression of anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL, and Mcl-1.[29-32] Among them, inhibition of constitutive STAT3 activation in malignant cells can suppress Bcl-xL or Mcl-1 genes.[29,30,33,34] We found that CT decreased Mcl-1 protein levels, but did not alter Bcl-xL levels in MC-3 cells. Interestingly, CT did not affect the expression level of Mcl-1 mRNA or its promoter activity indicating that Mcl-1 may not be regulated by CT at a transcriptional modification. Our previous reports revealed that several compounds such as tolfenamic acid and mithramycin A decreased Mcl-1 protein associated with proteasome-dependent protein degradation.[35,36] Other group also reported that Mcl-1 protein was rapidly degraded by the proteasome[37-39] suggesting that it could be modulated by posttranslational modification. Consistent with these findings, treatment with CT accelerated the clearance of Mcl-1 protein after the inhibition of protein synthesis by CHX and the inhibition of the proteasome function by MG-132 partially reversed the down-regulation of Mcl-1 by suggesting that CT does affect the stability of synthesized Mcl-1. The results suggest that CT clearly regulates survivin protein at a transcriptional level and alters Mcl-1 protein through the proteasome-dependent protein degradation.

To ensure that the *in vitro* activities seen in MC-3 cells are not cell line-specific, we examined the apoptotic effect of CT in YD-15, another human MEC cell line. The result showed that CT also can induce apoptotic cell death accompanied by inhibition of STAT3 phosphorylation in YD-15 cells. This suggests that STAT3 is a general molecular pathway that plays a critical role in CT-induced apoptosis in human MEC cell lines.

In summary, we have confirmed for the 1st time that CT is an effective apoptotic inducer through regulation

of the STAT3 signaling pathway in human MEC cell lines. Therefore, we suggest that CT may be a promising anticancer drug candidate for the treatment of MEC. However, further direction using an animal model is needed before clinical application of CT.

ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2012R1A2A2A01003731 and 2013R1A1A2057882).

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Cite this article as: Yu H, Park C, Kim S, Cho N, Cho S. Signal transducer and activators of transcription 3 regulates cryptotanshinone-induced apoptosis in human mucoepidermoid carcinoma cells. Phcog Mag 2014;10:622-9.

Source of Support: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A2A2A01003731 and 2013R1A1A2057882, **Conflict of Interest:** None declared.