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# Cryptotanshinone Prevents the Binding of S6K1 to mTOR/Raptor Leading to the Suppression of mTORC1-S6K1 Signaling Activity and Neoplastic Cell Transformation

Nam Ho Jeoung[1,2,*], Ji Yun Jeong[1,3,*], Bong Seok Kang[1]

[1]Bio-Medical Research Institute, School of Medicine, Kyungpook National University, Daegu, [2]Department of Pharmaceutical Engineering, Daegu Catholic University, Gyeongsan, [3]Department of Internal Medicine, Soonchunhyang University Gumi Hospital, Gumi, Korea

Cryptotanshinone is known for its inhibitory activity against tumorigenesis in various human cancer cells. However, exact mechanisms underlying the anticancer effects of cryptotanshinone are not fully elucidated. Here, we propose a plausible molecular mechanism, wherein cryptotanshinone represses rapamycin-sensitive mTORC1/S6K1 mediated cancer cell growth and cell transformation. We investigated the various effects of cryptotanshinone on the mTORC1/S6K1 axis, which is associated with the regulation of cell growth in response to nutritional and growth factor signals. We found that cryptotanshinone specifically inhibited the mTORC1-mediated phosphorylation of S6K1, which consequently suppressed the clonogenicity of SK-Hep1 cells and the neoplastic transformation of JB6 Cl41 cells induced by insulin-like growth factor-1. Finally, we observed that cryptotanshinone prevented S6K1 from binding to the Raptor/mTOR complex, rather than regulating mTOR and its upstream pathway. Overall, our findings provide a novel mechanism underlying anti-cancer effects cryptotanshinone targeting mTORC1 signaling, contributing to the development of anticancer agents involving metabolic cancer treatment.

**Key Words** mTORC1, p70S6K, neoplastic cell transformation, cryptotanshinone, Raptor protein

## INTRODUCTION

Mammalian target of rapamycin (mTOR) plays a central role in a nutrient-sensing signaling network that regulates the cell metabolisms and the insulin/IGF-1-dependent cell growth [1]. Two mTOR complexes (rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2) have been classified in mammalian cells [2]. mTOR regulates cell growth by interacting with Raptor, GβL, and PRAS40 to form mTORC1 [3,4]. In response to growth factors and nutrients, mTORC1 phosphorylates p70 S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) to stimulate ribosome biogenesis and protein synthesis [3,5], whereas mTORC2 phosphorylates AKT and PKC to control cell survival and cytoskeleton organization [6-8]. In hepatocellular carcinoma (HCC), the major pathways involved in carcinogenesis are WNT/β-catenin, VEGF, mitogen-activated protein kinase (MAPK) and PI3K/AKT/mTOR. Among them, the mTOR pathway is particularly interesting because it is constitutively activated in HCC patients and it is associated with a more aggressive tumor progression [9,10]. Therefore, mTOR, a central component of a signaling pathway that coordinates cell growth and nutrient availability, is a promising target for the treatment of metabolic diseases including cancer [11].

Tanshinones are a family of lipophilic phenanthrene compounds including tanshinone I, tanshinone IIA, and dihydrotanshinone. Cryptotanshinone (CT) is one of the major tanshinones extracted from *Salvia miltiorrhiza* (Danshen) [12] with biological properties including anti-bacterial, anti-oxidant, and anti-inflammatory activity [13-15]. Increasing evidence

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**Correspondence to** Bong Seok Kang, E-mail: kangx048@gmail.com, https://orcid.org/0000-0003-4040-8791
*These authors contribute equally to this work as co-first authors.

suggests that CT displays potent anti-cancer activity in human cancer cells, including prostate cancer [16,17], bladder cancer [18], and gastric cancer [19]. It has been reported that CT exerts anti-cancer effects by targeting mTOR signaling [16,17,20]. However, it remains unclear how this compound inhibits cancer cell growth via mTOR signaling.

The present study was aimed at exploring the detailed mechanism by which CT interacts with mTOR-S6K1 signaling to inhibit cancer cell growth and cell transformation. Our findings illustrated that CT displayed potent antitumor activity against the mTORC1/S6K1-activated SK-Hep1 liver cancer cells. These results have therefore expanded our knowledge on mTORC1-S6K1 regulation and indicate that mTOR/Raptor-S6K1 binding is a promising therapeutic target in the treatment of cancer and other metabolic diseases.

## MATERIALS AND METHODS

### Cell lines and reagents

SK-Hep1 cells derived from human HCC were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies Corporation, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies Corporation). JB6 Cl41 mouse epidermal cells were grown in minimal Eagle's medium (MEM; Gibco, Life Technologies Corporation) supplemented with 5% FBS. Tanshinone I (TI), tanshinone IIA (TIIA), and cryototanshinone (CT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin was purchased from EMD Biosciences (San Diego, CA, USA). Human insulin-like growth factor-1 (IGF-1) was purchased from Invitrogen (Carlsbad, CA, USA). All unspecified materials were purchased from Sigma-Aldrich.

### Cell viability assay

To estimate cell cytotoxicity, SK-Hep1 cells were seeded in 96-well plates ($1 \times 10^4$ cells/well) in 100 μL of DMEM supplemented with 10% FBS, and incubated at 37°C in a 5% $CO_2$ incubator. Following culture for 24 h, the cells were treated with various concentrations of TI, TIIA, and CT, and incubated for an additional 24 hours at 37°C in 5% $CO_2$. Next, 20 μL of the CellTiter 96® Aqueous One Solution (Promega, Madison, WI, USA) was added to each well and the plates were incubated for 3 hours at 37°C in 5% $CO_2$. Absorbance was measured at 490 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Immunoblotting and immunoprecipitation

The cells (SK-Hep1 or JB6 Cl41 cells) were pretreated with different doses of CT for 6 hours and then stimulated with 10% FBS or IGF-1 (10 ng/mL) for 30 minutes. To examine protein expression, SK-Hep1 cells ($4\text{-}5 \times 10^5$ cells) were cultured in their corresponding media in 60-mm dishes. Harvested cells were lysed in a buffer solution containing 40 mM HEPES (pH 7.4), 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1.5 mM $Na_3VO_4$, 10 mM β-glycerophosphate, 0.3% CHAPS, and EDTA-free protease inhibitors (Roche, Indianapolis, IN, USA). Cell lysates were mixed with 5× SDS sample buffer [0.25 M Tris-HCl (pH 6.8), 10% SDS, 25% glycerol, and bromophenol blue] containing 0.25 M dithiothreitol (DTT) and incubated for 5 minutes at 95°C. Proteins were resolved by SDS-PAGE, transferred to immune-blot® polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), and detected with ECL western blotting detection reagents (Perkin-Elmer, Wellesley, MA, USA). All primary antibodies for western blot analysis were purchased from Cell Signaling Technology (Danvers, MA, USA) and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunoprecipitation (IP) assays were carried out as described previously [21]. Briefly, whole-cell extracts were prepared in 400 μL of lysis buffer. The clarified supernatant fractions containing equal amounts of protein (500 μg) were subjected to IP followed by western blot analysis. mTOR antibody for IP assays was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). To investigate whether CT inhibited S6K1 phosphorylation by affecting the association of mTOR/Raptor with S6K1, mTOR was immunoprecipitated from IGF-1-stimulated SK-Hep1 cell lysate following pretreatment with CT, and immunoblotting was performed using mTOR, Raptor, GβL, and S6K1.

### Neoplastic cell transformation assay (soft agar assay)

The inhibition of clonogenicity in SK-Hep1 cells by CT was tested via a soft agar assay. Cells were grown in 0.3% basal medium Eagle's (BME) agar containing 10% FBS with cryptotanshinone (0, 3, 6, 12, and 25 μM) at $8 \times 10^3$ cells/mL.

IGF-1-induced anchorage-independent cell transformation assays were used to analyze JB6 Cl41 cells. In brief, cells ($4 \times 10^5$ cells) were plated in 6-cm dishes, following cultured in DMEM with 10% FBS at 37°C. After incubation for 24 hours, the cells were pre-treated with CT at the indicated concentration for 20 hours. The cells were then collected by trypsinization and subjected to a soft agar assay. The cells ($8 \times 10^3$ cells) were exposed to IGF-1 (10 ng/mL) in 1 mL of 0.3% basal medium Eagle's (BME) agar containing 10% FBS. The cultures were maintained at 37°C, in a 5% $CO_2$ incubator for 10 to 14 days, and the cell colonies were scored using an inverted microscope and the Image-Pro PLUS software program, as described previously [21,22].

### Statistical analysis

All data were expressed as the mean±SD. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical differences were evaluated using the One-way ANOVA with Bonferroni's multiple comparison test and considered significant at $P < 0.05$.

## RESULTS

### CT specifically inhibited the mTOR-mediated phosphorylation of S6K1

To assess the anti-tumor effect of TI, TIIA, and CT in SK-Hep1 cells, we first examined cell viability following treatment with each compound using the MTS assay. As shown in Figure 1A, CT and TI significantly inhibited the growth of HCC cells (SK-Hep1) in a dose-dependent manner, whereas TIIA marginally inhibited cell growth at high concentrations (> 100 μM). The data indicate that TI and CT strongly inhibited tumor cell growth. As mTORC1-S6K1 plays a critical role in cell growth, proliferation, and survival [2,4], we next examined whether the major tanshinones, including TI, TIIA and CT, affected the mTORC1 signaling pathways. Interestingly, only CT strongly inhibited the mTOR-mediated phosphorylation of S6K1 whilst the CT analogs TI and TIIA failed to (Fig. 1B). We further confirmed that CT did not inhibit the phosphorylation state of 4E-BP1, a downstream effector molecule of mTORC1, by using an antibody against 4E-BP1 (Fig. 1B). Phosphorylated 4E-BP1 showed decreased electrophoretic mobility during SDS-PAGE [23]. By contrast, all tested tanshinones did not affect the FBS-induced phosphorylation of upstream kinases of S6K1 such as ERKs, AMPKα and mTOR (Fig. 1C). These findings strongly suggest that, among the major tanshinones, CT specifically inhibits the mTORC1-S6K1 signaling axis, resulting in suppressed SK-Hep1 cell growth.

### CT suppressed the clonogenicity of SK-Hep1 cells

Based on results showing that CT can inhibit cell growth and S6K1 phosphorylation (Fig. 1), we hypothesized that it might also inhibit the clonogenicity of SK-Hep1 cells, and explored this possibility by first performing a soft agar clonogenic assay. The results showed that CT significantly inhibited the clonogenicity of SK-Hep1 cells in a concentration-dependent manner (Fig. 2A right panel). The colony size of cells treated with CT (3, 6, 12, 25 μM) was markedly reduced compared to that of control cells (DMSO) and cells treated with 0.1 μM rapamycin (Fig. 2A). Supporting observations that verify the inhibitory effect of CT on tumor cell growth were made during mTORC1-S6K1 signaling analysis, wherein CT inhibited the mTOR/Raptor-S6K1 signaling induced by FBS in SK-Hep1 cells. The results indicate that treatment of serum-starved SK-Hep1 cells with CT for 6 h inhibited the FBS-stimulated phosphorylation of S6K1 in a dose-dependent manner (Fig. 2B), consistent with the effect of CT shown in Figure 1. We

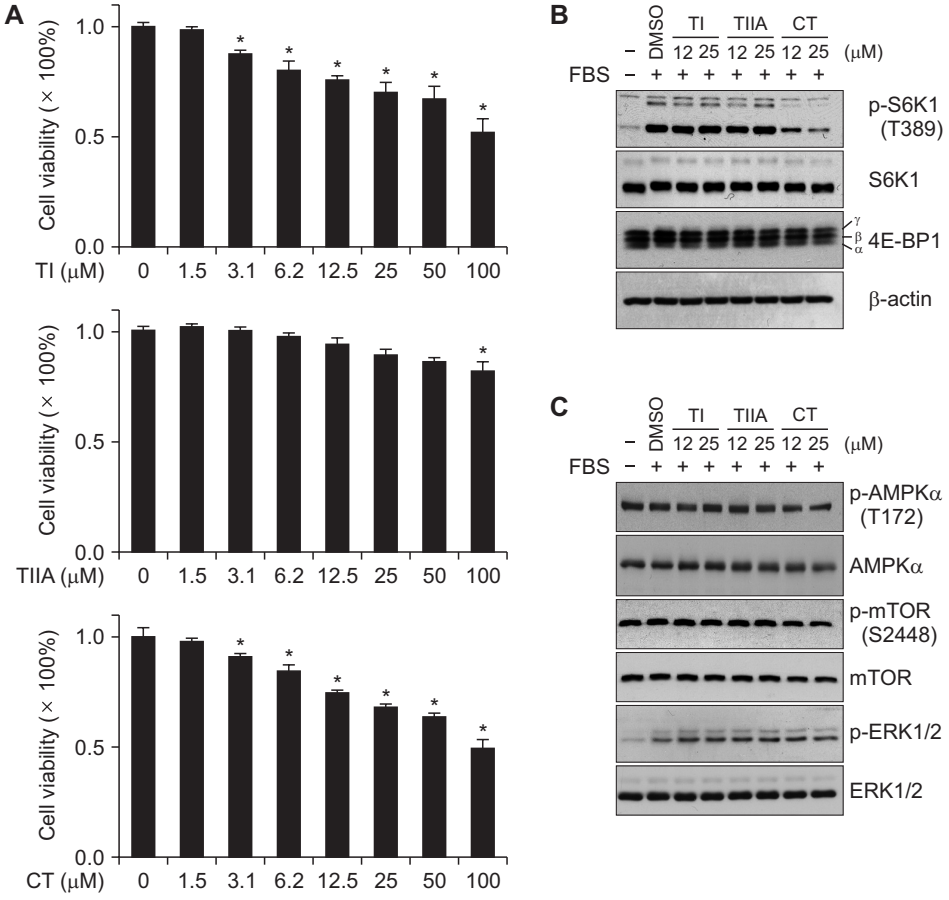


**Figure 1. Cryptotanshinone inhibits mTORC1 signaling and cell growth in cancer cells.** (A) Effects of three tanshinones on cell viability in SK-Hep1 cells (hepatocellular carcinoma cells). Cell viability was estimated. The asterisk (*) indicates a significant (*$P$ < 0.05) change compared to the untreated control. (B, C) Serum-starved SK-Hep1 cells were treated with three tanshinones (at 12 and 25 μM) for 6 hours, followed by immunoblotting with the corresponding antibodies. TI, Tanshinone I; TIIA, Tanshinone IIA; CT, Cryptotanshinone.

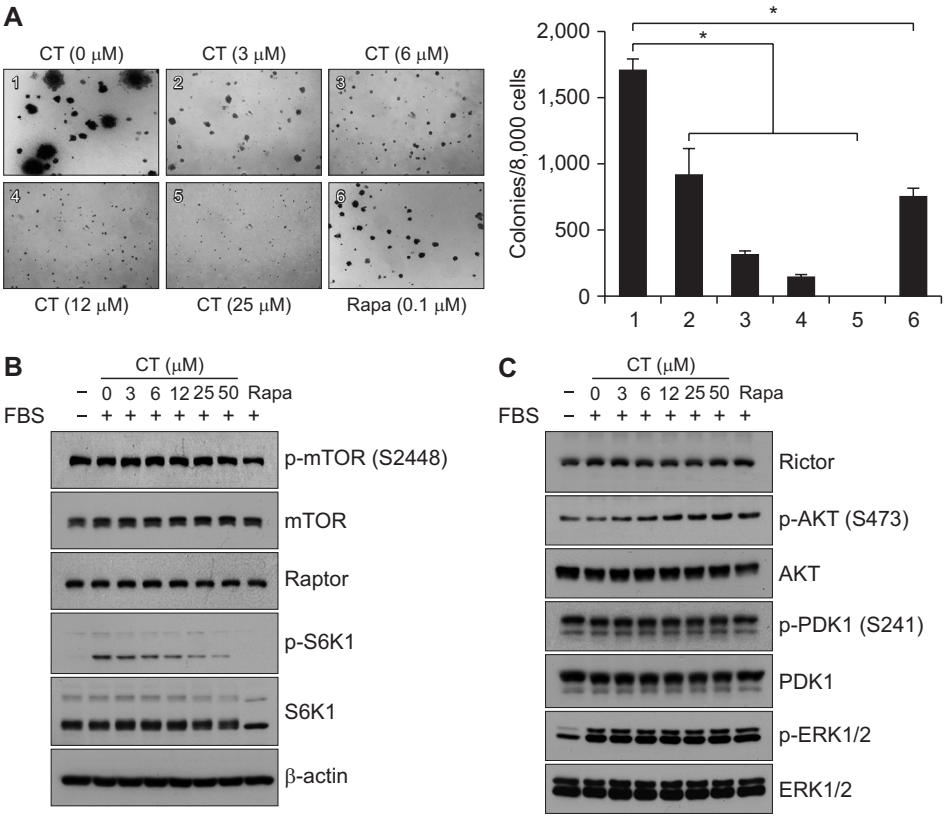


**Figure 2. Cryptotanshinone inhibits the clonogenicity of SK-Hep1 cells.** (A) Effect of CT on the clonogenicity of human hepatocellular carcinoma cells. SK-Hep1 cells were subjected to a soft agar clonogenic assay with cryptotanshinone (CT) or rapamycin (Rapa). Data are represented as means ± SD from three experiments (*$P$ < 0.05). (B, C) Cells were starved without serum for 24 hours and were either treated or not treated with the indicated concentration of CT and Rapa (0.1 μM) for an additional 6 hours. Next, the cells were stimulated with 10% FBS-DMEM. Cell lysates were subjected to immunoblotting assays with the indicated antibodies.

further clarified the effect of CT on the mTORC1 signaling pathway by focusing on the up- and down-stream effectors of mTORC1 in SK-Hep1 cells. The phosphorylation of mTOR and 3-phosphoinositide-dependent kinase 1 (PDK1) remained unaffected by treatment with CT (Fig. 2B and 2C). Next, we examined whether CT inhibited the mTORC2-mediated phosphorylation of AKT and observed that CT increased the AKT phosphorylation in SK-Hep1 cells in a concentration-dependent fashion (Fig. 2C). Under the same experimental conditions, there was no obvious effect of CT on the total protein levels of mTOR, S6K1, Raptor, and Rictor. These results indicate that the inhibitory effect of CT on the clonogenicity of SK-Hep1 cells was mainly due to its inhibition of the mTORC1-mediated S6K1 phosphorylation, but not mediated through inhibition of upstream signals of the mTORC1.

## CT inhibited the IGF-1-induced neoplastic cell transformation of JB6 Cl41 cells

To extend the results confirming the clonogenicity of SK-Hep1 cells, we performed neoplastic cell transformation assays using the JB6 Cl41 cell line. The JB6 Cl41 cell transformation is a well-developed model for studying tumor promotion induced by a tumor promoter (e.g., EGF, IGF-1, and TPA) under anchorage independent growth conditions [24,25]. Furthermore, mTOR signaling can also be activated by nutrients (e.g., amino acids) and growth factors (e.g., insulin, EGF, and IGF-1) [26]. Our findings also showed that CT significantly inhibited IGF-1-induced neoplastic cell transformation of JB6 Cl41 cells in a concentration-dependent manner (Fig. 3A). Further observations to supplement the results of the soft agar clonogenic assay were made in mTORC1-S6K1 signaling analysis, wherein CT inhibited the mTOR/Raptor(mTORC1)-S6K1 signaling pathway induced by IGF-1 in JB6 Cl41 cells. CT specifically inhibited S6K1 phosphorylation without inhibiting the mTOR phosphorylation, in a concentration-dependent manner, but did not alter other upstream signals of mTOR such as PDK1 and ERK (Fig. 3B and 3C). Notably, CT did not affect the mTORC2-mediated phosphorylation of AKT induced by IGF-1 in JB6 Cl41 cells (Fig. 3C). Collectively, these data strongly indicate that CT plays an inhibitory role in the neoplastic cell transformation of JB6 Cl41 cells stimulated with IGF-1, as well as in the clonogenicity of SK-Hep1 cells through inhibition of mTORC1-S6K1 signaling pathways.

## CT prevented the binding of S6K1 to mTOR/Raptor

To further investigate the mechanisms underlying the anticancer activity of CT, we examined the effect of CT on mTOR signaling using IGF-1-stimulated SK-Hep1 cells. After observing that CT significantly inhibited S6K1 phosphorylation in SK-Hep1 cells (Fig. 4A), we also tested whether it could inhibit the activity of upstream kinases such as PDK1, AMPKα, AKT, or ERK. Results showed that the increased CT concentration did not alter the phosphorylation of PDK1, AMPKα, and

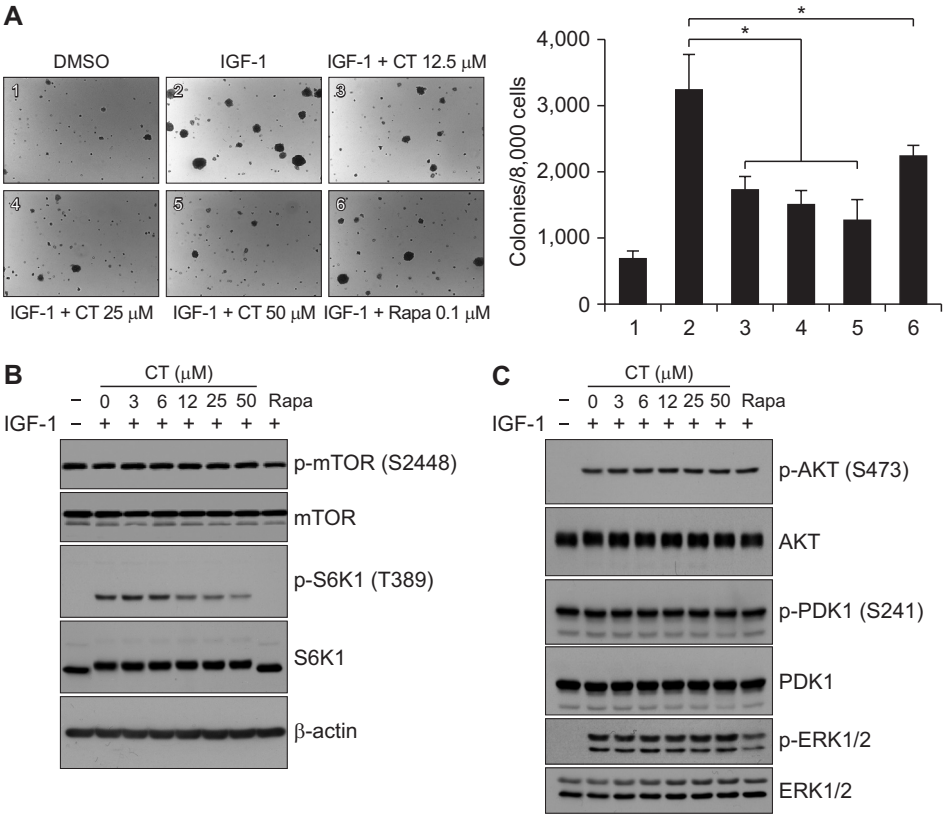


**Figure 3. Cryptotanshinone inhibits the IGF-1-induced neoplastic transformation of JB6 Cl41 cells.** (A) JB6 Cl41 cells were pre-treated with cryptotanshinone (CT) or rapamycin (Rapa) at the indicated concentrations for 20 hours. The cells were then collected by trypsinization and subjected to the soft agar assay. Data are represented as means ± SD from three experiments (*$P$ < 0.05). (B, C) JB6 Cl41 cells were starved without serum for 24 hours and were either treated or not treated with the indicated concentration of CT or Rapa (0.1 μM) for an additional 6 hours. Next, the cells were stimulated with IGF-1 (10 ng/mL). Cell lysates were subjected to immunoblotting assays with the indicated antibodies.

ERK1/2 induced by IGF-1 (Fig. 4A and 4B). However, CT increased the phosphorylation of AKT (S473), which is mediated by mTORC2, with increasing concentrations (Fig. 4B). These data suggest that CT could only inhibit mTORC1-mediated S6K1 signaling within the whole mTOR signal networks.

These findings prompted us to determine how CT inhibited the mTORC1-S6K1 signaling pathway induced by growth factors such as IGF-1. Based on the results from the mTORC1 signaling assay (Fig. 2, 3, 4A, and 4B), we hypothesized that CT might block the binding of S6K1 to mTORC1, leading to inhibition of S6K1 phosphorylation. Typically, rapamycin inhibits mTORC1 activity by dissociating Raptor from mTOR [4]. Consistent with previous findings, rapamycin, used as a positive control in the present study, dissociated Raptor from mTOR (Fig. 4C, lane 8). Notably, CT did not affect the binding of Raptor or GβL to the mTOR but inhibited the binding of S6K1 to the mTOR/Raptor/GβL complex (mTORC1) in a concentration-dependent manner (Fig. 4C). Collectively, these results strongly suggest that CT inhibits tumor cell growth by preventing the formation of a complex between S6K1 and mTOR/Raptor, which would typically be induced by growth factors such as serum and IGF-1 (Fig. 4D).

## DISCUSSION

The mTOR pathway is a key target for the chemoprevention and treatment of cancer and several other diseases, because of its pivotal role as a central hub in regulating numerous key cellular processes [2,11,27]. CT is the most active single anticancer factor found in Danshen. Several reports have shown that CT targets specific signaling pathways, that are responsible for regulating cell survival, cell cycle, and apoptosis [28]. CT inhibits cell proliferation, migration, and invasion in bladder cancer and activates the apoptosis via modulation of the PTEN/PI3K/AKT pathway [18]. In addition, CT also inhibits the mTORC1 signaling pathway by activating the AMPK-TSC2 axis, rather than directly inhibiting mTORC1 in the Rh30 (human rhabdomyosarcoma) and DU-145 (human prostate cancer) cell lines [20]. These findings suggest that CT indirectly inhibits the mTOR signaling and the subsequent downstream signaling, resulting in induced cell cycle arrest and apoptosis. Although several investigations explaining the anticancer activities of CT have been reported in cell culture studies, its own mechanism of action remains only partly understood.

To identify the mechanism by which CT inhibits mTOR signaling in cancer cells, we first focused on the mTOR and its upstream kinases, because CT typically inhibits the mTORC1-mediated phosphorylation of S6K1. mTOR/S6K1 signaling can be regulated either by the AMPK-TSC

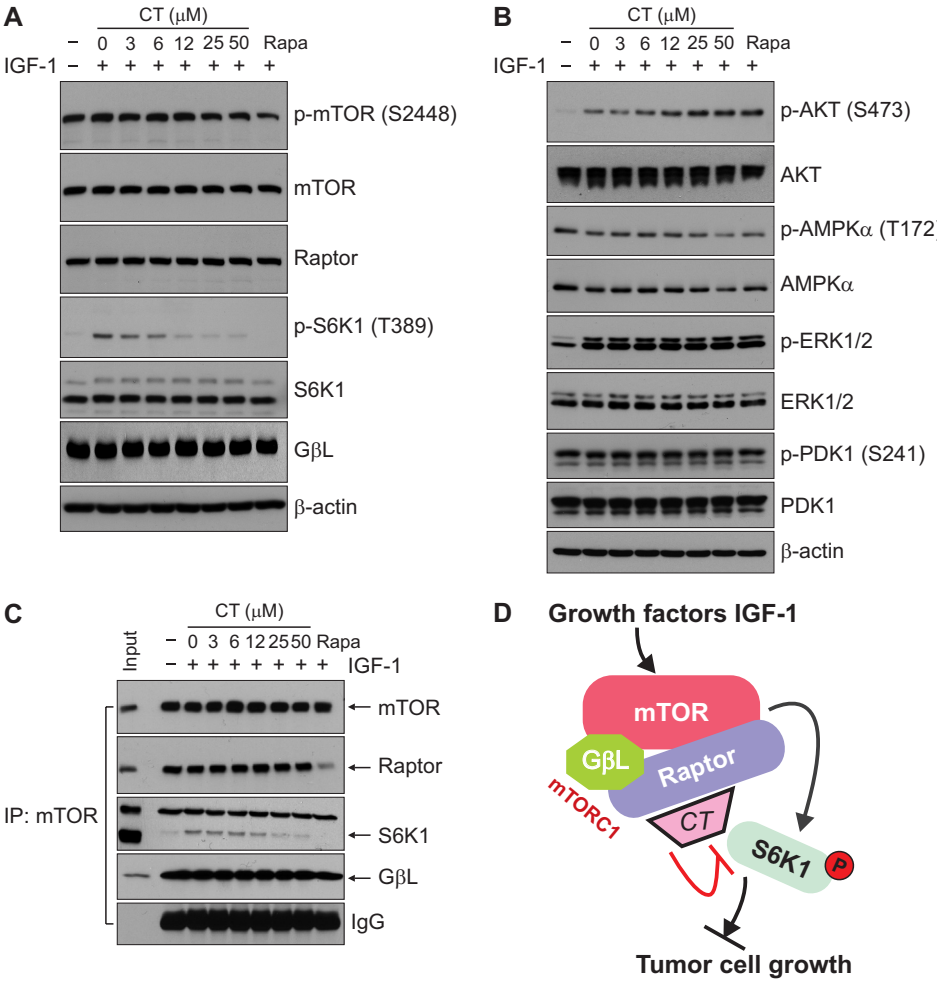


**Figure 4. Cryptotanshinone can prevent the binding of S6K1 to mTOR/Raptor induced by IGF-1.** (A, B) Effect of cryptotanshinone (CT) on IGF-1-induced mTOR signaling in SK-Hep1 cells. Serum-starved SK-Hep1 cells were treated with the indicated concentrations of CT or rapamycin (Rapa; 0.1 μM) for 6 hours, followed by stimulation with IGF-1 (10 ng/mL). Cells were then disrupted in a 0.3% CHAPS buffer. Cell lysates were subjected to either an immunoblotting assay as indicated for mTOR signaling (panels A and B) or to an immunoprecipitation (IP) assay with anti-mTOR (IP: mTOR, panel C). (C) Effect of CT on IGF-1-induced mTORC1-S6K1 complex formation. Proteins (500 μg) were used for immunoprecipitation with anti-mTOR and analyzed via immunoblotting with Raptor, GβL, and S6K1 antibodies, as indicated. The cell lysates used in the immunoprecipitation reactions were loaded as a positive control (Lysate). (D) Schematic diagram of the model hypothesizing the CT effect on the regulation of S6K1 phosphorylation by mTORC1.

or PDK1-AKT network [2], and can also be activated by the Ras-ERK1/2 network [29,30]. Our data indicate that CT inhibits mTORC1-S6K1 signaling not by affecting the upstream kinases such as PDK1/AKT, AMPKα, and ERK1/2. We found that CT increased the phosphorylation of AKT (S473) in SK-Hep1 cells. A previous study also showed that CT increased AKT (S473) phosphorylation in a concentration-dependent manner [16], which was consistent with our results. It has been described that S6K1 phosphorylates insulin receptor substrate-1 (IRS-1) to avoid hyperactivation of mTORC1 [31]. The inhibition of S6K1 activity may, therefore, prevent IRS-1 phosphorylation, resulting in accumulation of IRS-1 and activation of its downstream kinases by a feedback regulation mechanism. These phenomena suggested that CT might activate AKT via rapamycin-insensitive mTORC2 signaling by feedback regulation. Whether CT activates AKT through a feedback regulation mechanism remains to be elucidated.

Recent findings have revealed that CT could directly activate the AMPK-TSC2 axis, leading to the inhibition of mTORC1 signaling in some cancer cells [18]. By contrast, our results indicated that CT could not activate AMPK signaling, suggesting that AMPK was not involved in regulating of mTORC1 signaling in SK-Hep1 and JB6 Cl41 cells. We consequently confirmed the inhibitory effect of CT on the clonogenicity of SK-Hep1 liver cancer cells and the neoplastic transformation of JB6 Cl41 cells using anchorage-independent soft agar assays. In contrast to other previous studies [18,20,28], our results indicated that CT did not affect the upstream pathway of mTORC1 signaling, including the phosphorylation of mTOR itself. It is likely that CT might adopt a different mechanism to directly inhibit S6K1 phosphorylation.

We next investigated whether mTORC1 itself could act as the possible target of CT. To help explain the inhibitory role of CT in mTOR-mediated S6K1 phosphorylation, we also compared the effect of CT on mTORC1-S6K1 complex associations via the co-immunoprecipitation of mTOR. Finally, we confirmed that CT prevented the complex formation between S6K1 and mTORC1 without consequently disrupting the Raptor-mTOR association in mTORC1. This inhibitory effect on the functional association between S6K1 and mTORC1 was dependent on the CT concentration.

Previous reports have revealed that Raptor binds to the mTOR substrate S6K1 via its TOR signaling (TOS) motif, and that a loss of the TOS motif abolishes S6K1 phosphorylation

by mTORC1 [32]. The results obtained from mTOR co-immunoprecipitation experiments suggest that CT may bind to various sites including the TOS motif and TOS binding site, thereby inhibiting the Raptor-S6K1 binding. Further studies are required to determine how CT prevents S6K1 from binding to mTORC1. Several reports [18-20,33] have indicated that CT could be a potent drug candidate in the treatment of a broad range of cancers. Our findings also provide therapeutic potential of CT for cancer therapies in the context of its inhibition of the S6K1 binding to mTOR/Raptor.

In conclusion, our results showed that CT inhibited S6K1 phosphorylation by preventing the binding of S6K1 to mTOR/Raptor. Based on these results, we hypothesized (Fig. 4D) that the inhibition of functional binding between mTORC1 and S6K1 by CT has a profound effect on repressing rapamycin-sensitive mTORC1/S6K1 mediated cancer cell growth and cell transformation. Compared with previous studies, our findings provide a better understanding of the therapeutic paradigm for repressing the mTOR signaling activity and highlight the potential for developing specific inhibitors of the mTORC1-S6K1 signaling axis.

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## CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

## ORCID

Nam Ho Jeoung, https://orcid.org/0000-0002-7584-3761
Ji Yun Jeong, https://orcid.org/0000-0002-2052-7622
Bong Seok Kang, https://orcid.org/0000-0003-4040-8791

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