

Research Article

Cryptotanshinone Inhibits Cancer Cell Proliferation by Suppressing Mammalian Target of Rapamycin–Mediated Cyclin D1 Expression and Rb Phosphorylation

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Abstract

Cryptotanshinone (CPT), a natural compound isolated from the plant *Salvia miltiorrhiza* Bunge, is a potential anticancer agent. However, little is known about its anticancer mechanism. Here, we show that CPT inhibited cancer cell proliferation by arresting cells in G₁-G₀ phase of the cell cycle. This is associated with the inhibition of cyclin D1 expression and retinoblastoma (Rb) protein phosphorylation. Furthermore, we found that CPT inhibited the signaling pathway of the mammalian target of rapamycin (mTOR), a central regulator of cell proliferation. This is evidenced by the findings that CPT inhibited type I insulin-like growth factor I- or 10% fetal bovine serum-stimulated phosphorylation of mTOR, p70 S6 kinase 1, and eukaryotic initiation factor 4E binding protein 1 in a concentration- and time-dependent manner. Expression of constitutively active mTOR conferred resistance to CPT inhibition of cyclin D1 expression and Rb phosphorylation, as well as cell growth. The results suggest that CPT is a novel anti-proliferative agent. *Cancer Prev Res*; 3(8); 1015–25. ©2010 AACR.

Introduction

Deregulation of cell cycle progression plays an important role in the development of cancer (1). Cell cycle progression occurs in an orderly fashion and is tightly regulated by the expression and activity of cyclin/cyclin-dependent kinase (CDK) complexes (1, 2). The CDK complexes can be activated at specific points of the cell cycle when a CDK is bound with cyclins, or inactivated when CDK inhibitors bind with the CDK (1, 2). Of cyclins, cyclin D1 is critical in controlling cell cycle progression from G₁ to S phase (2, 3). Cyclin D1 binds to CDK4/6 and forms the cyclin/CDK complexes, resulting in phosphorylation and activation of the CDKs (1). Subsequently, the activated CDKs phosphorylate the retinoblastoma (Rb) tumor suppressor protein (4). Hypophosphorylated Rb binds E2F, suppressing the transcription activity of E2F, whereas phosphorylated Rb is released from E2F, promoting transition

from G₁ to S phase (4). Therefore, the Rb protein has an essential role in mediating G₁ progression through the restriction point.

The mammalian target of rapamycin (mTOR), a Ser/Thr kinase, lies downstream of type I insulin-like growth factor (IGF-I) receptor-phosphatidylinositol 3'-kinase (PI3K; ref. 5). Recently, two mTOR complexes (mTORC1 and mTORC2) have been identified in mammalian cells (5). mTORC1 is composed of mTOR, mLST8 [also termed G-protein β-subunit-like protein (GβL), a yeast homologue of LST8], PRAS40 (proline-rich Akt substrate 40 kDa), and raptor (regulatory-associated protein of mTOR) and is rapamycin sensitive (6–12). In response to growth factors and nutrients, mTORC1 regulates cell proliferation and growth by controlling protein (cyclin D1, ornithine decarboxylase, etc.) synthesis and ribosome biogenesis through phosphorylation of downstream effectors such as eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and ribosomal p70 S6 kinase 1 (S6K1; refs. 6–13). mTORC2 consists of mTOR, mLST8, mSin1 (mammalian stress-activated protein kinase-interacting protein 1), rictor (rapamycin-insensitive companion of mTOR), protor (protein observed with rictor), and PRR5 (proline-rich protein 5) and is rapamycin insensitive (14–20). mTORC2 phosphorylates Akt and protein kinase C, signals to small GTPases (RhoA and Rac1), and controls cytoskeleton organization (16, 21–24). Although the cellular functions of the mTOR complexes remain to be determined, current data indicate that mTOR is a key kinase in the regulation of cell proliferation, growth, survival, differentiation, motility, and angiogenesis (5, 13).

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Cryptotanshinone (CPT) is one of the major tanshinones isolated from *Salvia miltiorrhiza* Bunge (Danshen) that has been used in traditional oriental medicine for the treatment of a variety of diseases including coronary artery disease (25), hyperlipidemia (26), acute ischemic stroke (26), chronic renal failure (27), chronic hepatitis (28), and Alzheimer's disease (29). Studies have shown that CPT inhibits inflammation by suppressing cyclooxygenase-2 activity (30); inhibits tumor necrosis factor- α -induced matrix metalloproteinase-9 production and migration in human aortic smooth muscle cells through downregulation of NF- κ B and activator protein-1 (31); and acts against diabetes and obesity by activating AMP-activated protein kinase (32). CPT inhibits chemotactic migration in macrophages by blocking PI3K signaling (33), but protects primary rat cortical neurons from glutamate-induced neurotoxicity by activating PI3K/Akt signaling (34). Most recent studies have shown that CPT is also a potential anticancer agent (35, 36). Although CPT has been found to inhibit prostate cancer cell growth by inactivating the signal transducer and activator of transcription-3 (Stat3) activity (36), the anticancer mechanism of CPT remains to be elucidated.

Here we show that CPT inhibited the growth of a panel of tumor cell lines by arresting cells in G₁-G₀ phase of the cell cycle. Concurrently, CPT inhibited the expression of cyclin D1 and the phosphorylation of Rb protein. Further, we found that this is related to inhibition of the mTOR signaling pathway.

Materials and Methods

Materials

CPT, tanshinone I, tanshinone IIA, and dihydrotanshinone were extracted from the roots of *S. miltiorrhiza* Bunge (Danshen) using ethanol. Briefly, the root (200 g) of Danshen or red sage (*S. miltiorrhiza*) was extracted with 2 liters of 95% aqueous ethanol in a high-power blending extractor for 10 minutes. After the extraction, the supernatant solution was filtered through a filter paper (Whatman #4). The filtrate was freed of ethanol under reduced pressure and lyophilized to powder (10.95 g). The ethanol extract yield was ~5.5% (w/w). High-pressure liquid chromatography fingerprints showed that the ethanol extract of Danshen contained numerous components, including the water-soluble salvianolic acid B and the water-insoluble tanshinones including the indicated four compounds. The four tanshinone compounds were purified by high-pressure liquid chromatography (>95% purity for dihydrotanshinone and >98% purity for CPT, tanshinone I, and tanshinone IIA) and were dissolved in 100% ethanol to prepare stock solutions (20 mmol/L), which were aliquoted and stored at -20°C.

RPMI 1640 and DMEM were purchased from Mediatech. Fetal bovine serum (FBS) was from Hyclone, and 0.05% trypsin-EDTA from Invitrogen. IGF-1 (PeproTech) was rehydrated in 0.1 mol/L acetic acid to prepare a stock solution (10 μ g/mL), aliquoted, and stored at -80°C. Enhanced chemiluminescence solution was from

Perkin-Elmer Life Science. CellTiter 96 AQueous One Solution cell proliferation assay kit was from Promega. The following antibodies were used: 4E-BP1 (Zymed); Akt, p-S6K1 (Thr389), S6K1, cyclin D1, Rb, p-Rb (Ser780), CDK2, and CDK4 (Santa Cruz Biotechnology); phospho-Akt (Ser473, Thr308), phospho-mTOR (Ser2448), and mTOR (Cell Signaling); AU1 (Bethyl Laboratories); β -tubulin (Sigma); and goat anti-mouse IgG-horseradish peroxidase and goat anti-rabbit IgG-horseradish peroxidase (Pierce).

Cell lines and cultures

The human rhabdomyosarcoma Rh30 cell line expressing mutant (R273C) *p53* alleles (37) was generously provided by Dr. Peter J. Houghton (St. Jude Children's Research Hospital, Memphis, TN). Human prostate carcinoma (DU145) and breast carcinoma (MCF-7) cells were from the American Type Culture Collection. Rh30 and DU145 cells were grown in antibiotic-free RPMI 1640 supplemented with 10% FBS, whereas MCF-7 cells were grown in antibiotic-free DMEM supplemented with 10% FBS. All cells were maintained in a humid incubator (37°C, 5% CO₂). For experiments where cells were deprived of serum, cell monolayers were washed with PBS and incubated in serum-free DMEM.

One Solution cell proliferation assay

Cell proliferation was evaluated using the CellTiter 96 AQueous One Solution cell proliferation assay (Promega), which is a colorimetric method to determine the number of viable cells in proliferation or cytotoxicity assays. Briefly, cells suspended in the growth medium were seeded in a 96-well plate at a density of 1×10^4 per well (in six replicates) and were grown overnight at 37°C in a humidified incubator with 5% CO₂. The next day, CPT, tanshinone I, tanshinone IIA, or dihydrotanshinone (0-40 μ mol/L) was added. After incubation for 48 hours, 20 μ L of One Solution reagent (Promega) were added to each well and incubation was continued for a further 4 hours. Cell proliferation was determined by measuring the absorbance at 490 nm using a Wallac 1420 multilabel counter (Perkin-Elmer Life Sciences).

[³H]Thymidine incorporation assay

[³H]Thymidine incorporation assay was done as described (38). Briefly, Rh30 or DU145 cells (3×10^4 per well) were seeded in 48-well plates in triplicate with 10% FBS-RPMI 1640 and were grown overnight at 37°C in a humidified incubator with 5% CO₂. The next day, CPT (0-40 μ mol/L) was added. After incubation for 48 hours, [methyl-³H]thymidine (1 μ Ci/well; Amersham Biosciences) was added. Following incubation for 8 hours at 37°C, the used medium was aspirated. Subsequently, the cells were briefly washed with cold PBS and then incubated with ice-cold 5% trichloroacetic acid for 30 minutes at 4°C. After a wash with PBS, the cells were incubated with 0.5 mol/L NaOH/0.5% SDS (250 μ L/well) for 1 hour. Finally, the activity of [³H]thymidine

incorporated was measured with a LS6500 scintillation counter (Beckman Coulter).

Cell cycle analysis

Cell cycle analysis was done as described previously (37). Briefly, cells were seeded in 100-mm dishes at a density of 1×10^6 per dish in the growth medium and were grown overnight at 37°C in a humidified incubator with 5% CO₂. After treatment with CPT (0-40 μmol/L) for 24 hours, the cells were briefly washed with PBS and trypsinized. Cell suspensions were centrifuged at 1,000 rpm for 5 minutes, and pellets were stained with Cellular DNA Flow Cytometric Analysis Kit (Roche Diagnostics Corp.). Percentages of cells within each of the cell cycle compartments (G₀-G₁, S, or G₂-M) were determined by flow cytometry (FACSCalibur, Becton Dickinson). Cells treated with vehicle alone (100% ethanol) were used as a control.

Expression of constitutively active mTOR by adenoviral infection of cells

Recombinant adenovirus encoding AU1-tagged constitutively active mTOR (Ad-mTOR-RD) was a gift from Dr. Christopher J. Rhodes (Pacific Northwest Research Institute, Seattle, WA; ref. 39). The virus was amplified and titrated as described previously (40). For the experiments, Rh30 cells were grown in six-well plates with RPMI 1640 supplemented with 10% FBS and infected with Ad-mTOR-RD for 24 hours at a multiplicity of infection of 5. Subsequently, cells were serum starved for 24 hours and then treated with or without CPT (20 μmol/L) for 2 hours, followed by stimulation with or without IGF-I (10 ng/mL) for 1 hour. Cells infected with Ad-GFP, encoding the green fluorescent protein (GFP; ref. 41), served as a control. Expression of AU1-tagged constitutively active mTOR was confirmed by Western blotting with antibodies to AU1, p-S6K1, 4E-BP1, and β-tubulin.

Western blot analysis

Western blotting was done as described previously (37). Briefly, following treatment, cells were washed with cold PBS. On ice, cells were lysed in radioimmunoprecipitation assay buffer, containing 50 mmol/L Tris (pH 7.2), 150 mmol/L NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton-X 100, 10 mmol/L NaF, 1 mmol/L Na₃VO₄, and protease inhibitor cocktail (1:1000; Sigma). Lysates were sonicated for 10 seconds and centrifuged at 14,000 rpm for 10 minutes at 4°C. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as standard (Pierce). Equivalent amounts of protein were separated on 7.5% to 12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were incubated with PBS containing 0.05% Tween 20 and 5% nonfat dry milk to block nonspecific binding and were incubated with primary antibodies, then with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized

by using Renaissance chemiluminescence reagent (Perkin-Elmer Life Science).

Statistical analysis

Results were expressed as mean ± SE. The data were analyzed by one-way ANOVA followed by post hoc Dunnett's *t* test for multiple comparisons. *P* < 0.05 was considered to be significant.

Results

CPT inhibits cancer cell proliferation

To assess the antiproliferative activities of CPT, tanshinone I, tanshinone IIA, and dihydrotanshinone CPT, One Solution assay was used. As shown in Fig. 1A, treatment with the compounds for 48 hours inhibited the growth of rhabdomyosarcoma (Rh30) and prostate cancer (DU145) cells in a concentration-dependent manner. Of the compounds, only CPT potently inhibited the growth of the cells at low micromolar concentrations (2.5-10 μmol/L). The IC₅₀ values of CPT were ~5.1 μmol/L in Rh30 and 3.5 μmol/L in DU145 cells, whereas the IC₅₀ values of dihydrotanshinone, tanshinone I, and tanshinone IIA were >20 μmol/L in both Rh30 and DU145 cells. Similar data were also observed in MCF-7 cells (data not shown). The data suggest that CPT, among the tanshinones, is the most potent antiproliferative agent.

Because the One Solution assay, in fact, determines the number of viable cells in proliferation or cytotoxicity assays, to confirm the inhibitory effects of CPT on cell proliferation, we have also performed [³H]thymidine incorporation assay. As shown in Fig. 1B, CPT inhibited [³H]thymidine incorporation in Rh30 and DU145 cells in a concentration-dependent manner. The IC₅₀ values of CPT were ~8.5 μmol/L in Rh30 and 5.2 μmol/L in DU145 cells. The data indicate that CPT inhibits tumor cell proliferation.

CPT arrests cells in G₁-G₀ phase of the cell cycle by inhibiting cyclin D1 expression and Rb phosphorylation

Cell proliferation is controlled by the progression of the cell cycle (1). To understand how CPT inhibits cell proliferation, the effect of CPT on cell cycle distribution was assessed using the Cellular DNA Flow Cytometric Analysis Kit (Roche Diagnostics) and flow cytometry. We found that treatment with CPT for 24 hours arrested Rh30 and DU145 cells in G₁-G₀ phase of the cell cycle in a concentration-dependent manner, consistent with the results shown in Fig. 1. At 10 μmol/L, CPT treatment significantly increased the proportion of Rh30 cells in the G₁-G₀ phase from 44.28% to 60.33% (Fig. 2A). This increase in G₁-G₀ cell population was accompanied with a concomitant decrease of cell number in S-phase or G₂-M phase of the cell cycle (Fig. 2B). Prolonged treatment (48 hours) with CPT did not significantly enhance the proportion of Rh30 cells in the G₁-G₀ phase (e.g., at 10 μmol/L, 60.33% for 24 hours versus 61.87% for 48 hours; data not shown). Similar

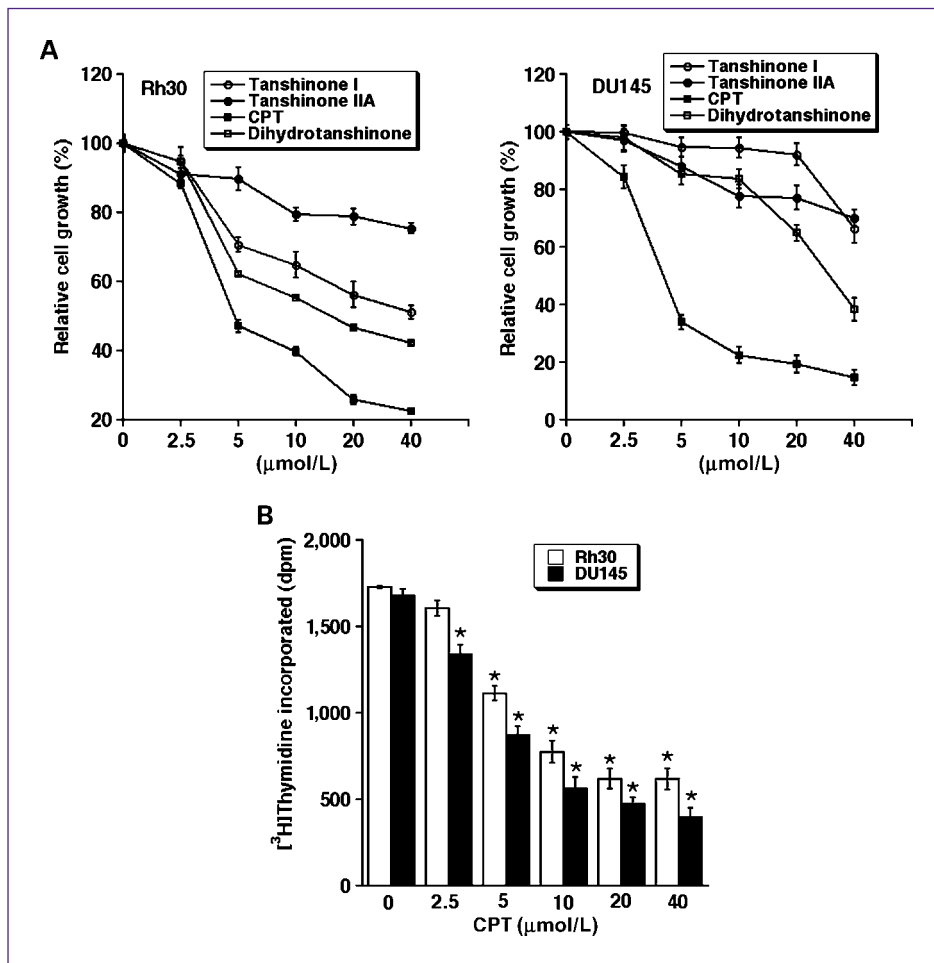


Fig. 1. CPT inhibits the proliferation of cancer cells in a concentration-dependent manner. Rh30 and DU145, grown in 96-well plates with RPMI 1640 supplemented with 10% FBS, were exposed to the indicated compounds (0–40 $\mu\text{mol/L}$) for 48 h. Cell proliferation was evaluated using One Solution cell proliferation assay (A) or [^3H] thymidine incorporation assay (B), as described in Materials and Methods. Points and columns, mean ($n = 3$); bars, SE. *, $P < 0.05$, versus control.

results were observed in DU145 cells, even when treated with CPT for up to 96 hours (see Supplementary Fig. S1), indicating that CPT inhibition of cell proliferation is attributed to the induction of G_1 - G_0 cell cycle arrest.

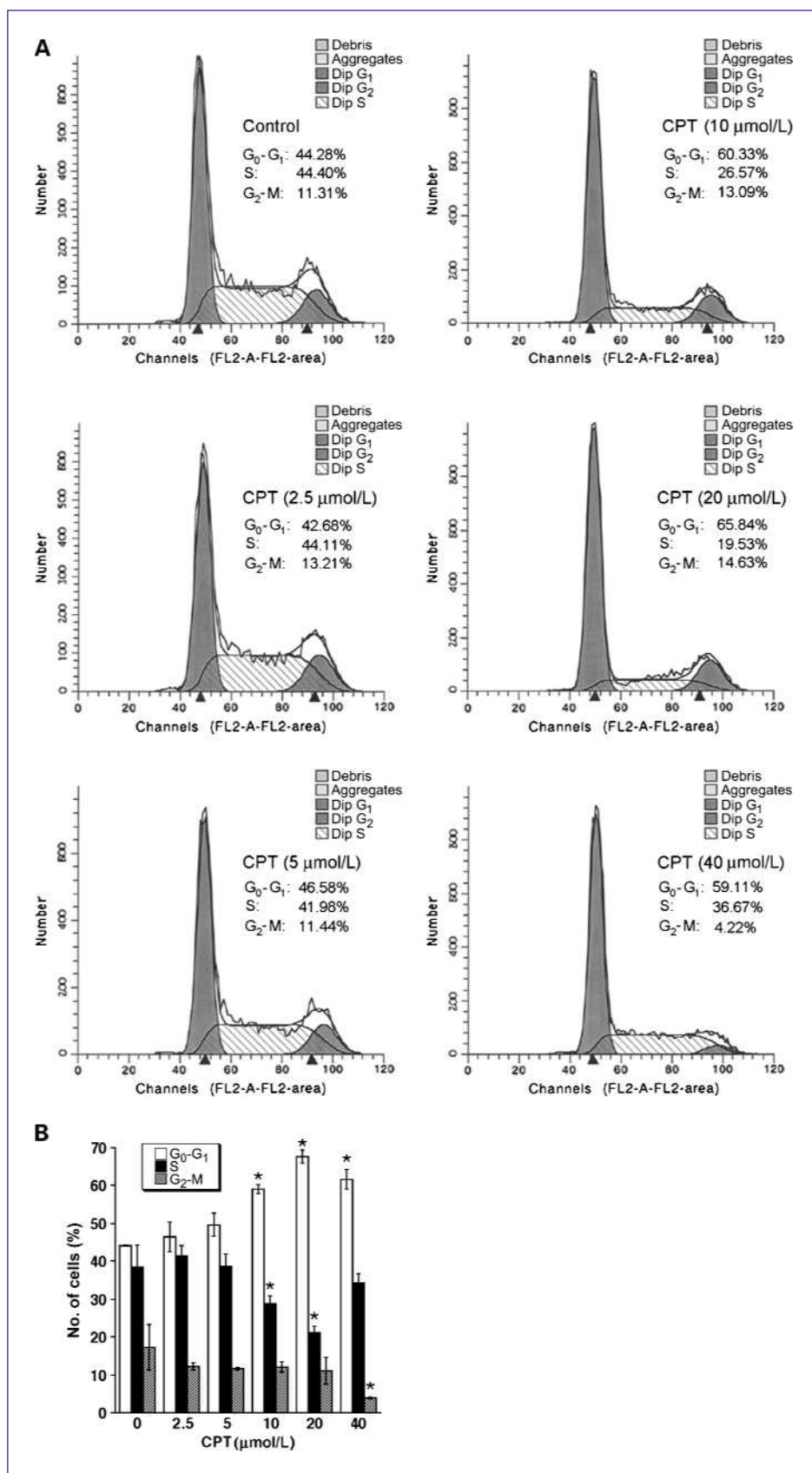
Cyclins and CDKs play an essential role in the regulation of cell cycle progression (1). Thus, perturbation of cyclin or CDK expression may contribute to altered cell cycle distribution. Of the cell cycle-related cyclins, cyclins D and E play an important role in the transition from the G_1 to the S phase, and cyclin D1-CDK4/6 and cyclin E-CDK2 complexes are required for G_1 progression (1). Because CPT induced G_1 - G_0 cell cycle arrest, we next examined the protein expression of cyclin A, cyclin B1, cyclin D1, cyclin E, CDK2, and CDK4. As shown in Fig. 3, treatment of Rh30 with CPT for 24 hours inhibited the cellular protein expression of cyclin D1 in a concentration-dependent manner. Starting at 10 $\mu\text{mol/L}$, CPT reduced the expression of cyclin D1 sharply. Protein levels of other molecules including cyclin A, cyclin B1, cyclin E, CDK2, and CDK4 were not obviously altered (Fig. 3). As Rb, one of the most important G_1 phase cyclin/CDK substrates, functions as an archetypal tumor suppressor and a regulator of cell cycle progression in the late G_1 phase

(2), we investigated the effect of CPT on Rb phosphorylation. As shown in Fig. 3, Rb was expressed as a 110-kDa band on Western blotting in vehicle-treated control Rh30 cells. After 10 $\mu\text{mol/L}$ CPT treatment for 24 hours, a lower band, which migrates rapidly and represents the dephosphorylated protein, was observed, indicating that CPT inhibits the phosphorylation of Rb (Fig. 3). The status of Rb phosphorylation is closely related to the level of cyclin D1. Similar results were observed in DU145 cells (see Supplementary Fig. S2). The data suggest that CPT arrests cells in G_1 - G_0 phase of the cell cycle by inhibiting cyclin D1 expression and Rb phosphorylation, which are not cell type dependent.

CPT inhibits the mTOR signaling pathway

mTOR plays a central role in the regulation of cell proliferation and growth (5, 13). Inhibition of mTOR by rapamycin downregulates cyclin D1 expression (42) and Rb phosphorylation (43), resulting in cell cycle arrest in G_1 - G_0 phase. Because we found that CPT arrested cells in G_1 - G_0 phase of the cell cycle by inhibiting cyclin D1 expression and Rb phosphorylation, we hypothesized that CPT may inhibit the mTOR signaling pathway.

Fig. 2. CPT arrests cell cycle in G_1 - G_0 phase in cancer cells. A and B, Rh30 cells, grown in six-well plates (1×10^5 per well) with RPMI 1640 supplemented with 10% FBS, were treated with CPT (0-40 $\mu\text{mol/L}$) for 24 h, followed by cell cycle analysis using the Cellular DNA Flow Cytometric Analysis Kit. A, representative cell cycle analysis results for Rh30 cells. B, columns, mean ($n = 3$); bars, SE. *, $P < 0.05$, versus control.



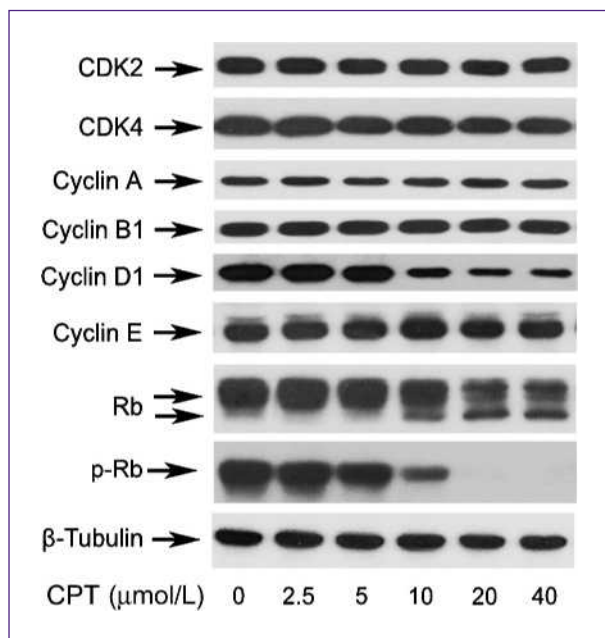


Fig. 3. CPT inhibits cyclin D1 expression and Rb phosphorylation in cancer cells. Rh30 cells were treated with CPT (0–40 $\mu\text{mol/L}$) for 24 h, followed by Western blotting with the indicated antibodies. β -Tubulin was used as a loading control.

To test this hypothesis, we set out to examine the effect of CPT on mTOR signaling in Rh30 and DU145 cells. As mTOR signaling can be activated by nutrients, hormones, and growth factors, such as insulin and IGF-I (5), we therefore first investigated the effect of CPT on mTOR signaling in IGF-I-stimulated cells. The results indicate that treatment of serum-starved Rh30 cells with CPT for 2 hours inhibited IGF-I-stimulated phosphorylation of S6K1 and 4E-BP1, the two best-characterized downstream effector molecules of mTORC1 (5), in a dose- and time-dependent manner (Fig. 4A). After 2 hours of exposure, CPT remarkably suppressed IGF-I-stimulated phosphorylation of S6K1 (Thr389) starting at 2.5 $\mu\text{mol/L}$ (Fig. 4A); at 10 $\mu\text{mol/L}$, CPT dramatically inhibited this phosphorylation event within 2 hours in Rh30 cells (Fig. 4A). No obvious effect of CPT on total protein levels of S6K1 was detected using an anti-S6K1 antibody that recognizes both phosphorylated and unphosphorylated forms (Fig. 4A). Similarly, the effect of CPT on the phosphorylation state of 4E-BP1 was detected with an antibody to 4E-BP1. Phosphorylation of 4E-BP1 decreases its electrophoretic mobility during SDS-PAGE (44). CPT inhibited IGF-I-stimulated phosphorylation of 4E-BP1 in Rh30 cells, as indicated by the decrease in the intensity of the uppermost band γ and by the increase in the higher mobility band α that corresponds to a less phosphorylated form of 4E-BP1 (Fig. 4A). In addition, we found that CPT also inhibited IGF-I-stimulated phosphorylation of mTOR at Ser2448, a site phosphorylated by S6K1 (45), in a dose- and time-dependent manner (Fig. 4A). Similar data were

also observed in DU145 and MCF-7 cells (data not shown) and in Rh30 cells grown in the normal culture medium containing 10% FBS (Fig. 4B). Moreover, we found that CPT analogues, including tanshinone I, tanshinone IIA, and dihydrotanshinone, did not obviously alter the phosphorylation of S6K1, 4E-BP1, and mTOR (Fig. 4C), which is consistent with the findings that CPT, but not tanshinone I, tanshinone IIA, and dihydrotanshinone, potentially inhibited cancer cell growth.

mTOR functions as two complexes, mTORC1 and mTORC2, which phosphorylate S6K1/4E-BP1 and Akt, respectively (16, 17, 21). After showing that CPT inhibits mTORC1-mediated phosphorylation of S6K1 and 4E-BP1, we further tested whether CPT inhibits mTORC2-mediated phosphorylation of Akt. To our surprise, CPT increased the phosphorylation of Akt (S473 and T308) in Rh30 cells and DU145 cells in a concentration-dependent manner (Fig. 5). Taken together, our data suggest that CPT may represent a novel inhibitor for mTORC1, but not for mTORC2.

Expression of constitutively active mTOR confers high resistance to CPT inhibition of mTOR signaling, cyclin D1 expression, and Rb phosphorylation

mTOR regulates cyclinD1 expression and Rb phosphorylation, and inhibition of mTOR by rapamycin arrests cells in G_1 - G_0 phase of the cell cycle (42, 43). To determine whether CPT inhibition of cyclin D1 expression and Rb phosphorylation is due to inhibition of mTOR signaling, Rh30 cells were infected with a recombinant adenovirus expressing AU1-tagged constitutively active mTOR (mTOR-RD). We found that ectopic expression of constitutively active mTOR increased the basal level of phosphorylation of S6K1, but not Akt, in serum-starved Rh30 cells (Fig. 6A), suggesting that the constitutively active mTOR was functional in the cells. Of interest, treatment with CPT (10 $\mu\text{mol/L}$) for 24 hours inhibited basal or IGF-I-stimulated S6K1 phosphorylation, as well as cyclin D1 expression and Rb phosphorylation, in the cells infected with Ad-GFP (Fig. 6A), which is consistent with the data seen in the parental Rh30 cells (Fig. 3). However, expression of constitutively active mTOR conferred high resistance to CPT inhibition of S6K1 phosphorylation, as well as cyclin D1 expression and Rb phosphorylation (Fig. 6A). The results suggest that CPT inhibits cyclin D1 expression and Rb phosphorylation by targeting mTOR signaling.

Expression of constitutively active mTOR partially prevents CPT inhibition of cancer cell growth

To further determine the role of mTOR in CPT inhibition of cell growth, Rh30 cells were infected with Ad-GFP (control) and Ad-mTOR-RD. Expression of constitutively active mTOR, but not GFP, rendered partial resistance to CPT inhibition of cell growth. This is evidenced by cell counting (Fig. 6B) and cell cycle analysis (Fig. 6C). Expression of constitutively active mTOR significantly prevented CPT (5–20 $\mu\text{mol/L}$) inhibition of cell growth by

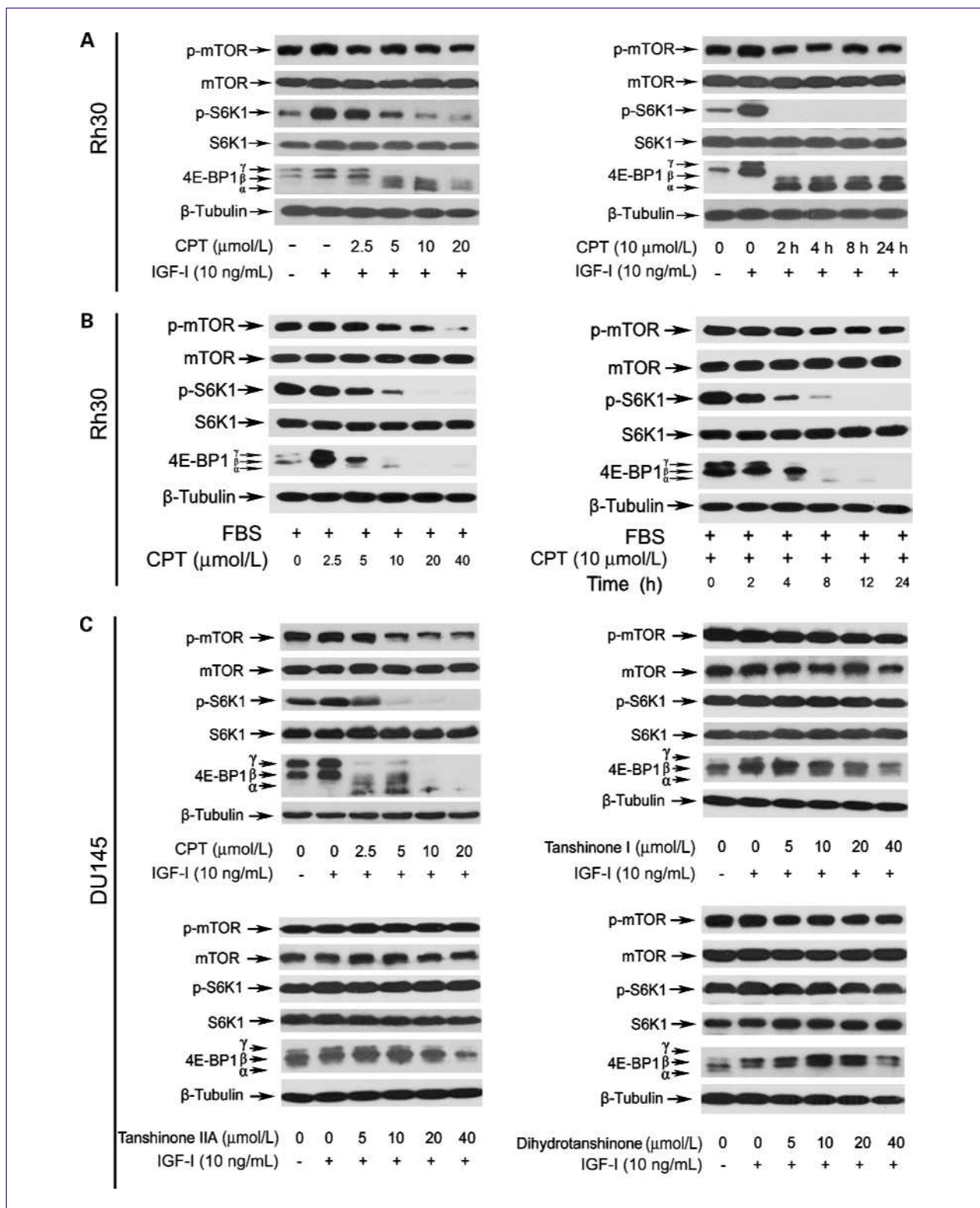


Fig. 4. CPT inhibits the mTOR pathway in cancer cells. A to C, Western blot results. β-Tubulin was used as a loading control. Serum-starved (A) or nonstarved Rh30 cells (B) were pretreated with CPT (0-40 μmol/L) for 2 h, and then stimulated with IGF-I (10 ng/mL) for 1 h (left) or with CPT (10 μmol/L) for the indicated time periods and stimulated with IGF-I (10 ng/mL) for 1 h (right). C, serum-starved DU145 cells were treated with CPT, tanshinone I, tanshinone IIA, or dihydro-Tanshinone (0-40 μmol/L) for 2 h and then stimulated with IGF-I (10 ng/mL) for 1 h.

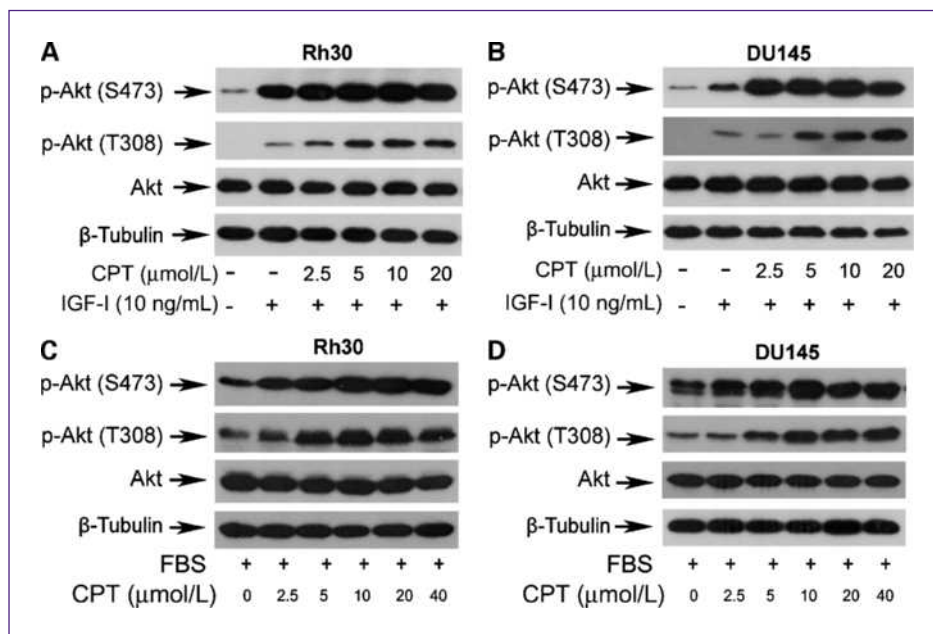


Fig. 5. CPT activates Akt in a concentration-dependent manner. Serum-starved (A and B) or nonstarved (C and D) Rh30 and DU145 cells were pretreated with CPT (0–40 μmol/L) for 2 h and then stimulated with (A and B) or without (C and D) IGF-I (10 ng/mL) for 1 h. The cell lysates were subjected to Western blot analysis with the indicated antibodies. β-Tubulin was used as a loading control.

1.4- to 2.2-fold (Fig. 6B). Cell cycle analysis reveals that expression of constitutively active mTOR also significantly attenuated CPT induction of G₁-G₀ cell cycle arrest (Fig. 6C). Similar data were observed in DU145 cells (see Supplementary Fig. S3). The results suggest that CPT inhibits cancer cell growth, at least partially, by inhibiting mTOR signaling.

Discussion

In this study, we observed that CPT inhibited cell proliferation by arresting the cell cycle in G₁-G₀ phase in rhabdomyosarcoma (Rh30), prostate (DU145), and breast (MCF-7) cancer cells. This is related to CPT arresting cells in G₁-G₀ phase by inhibiting the expression of cyclin D1 and the phosphorylation of Rb protein. Of importance, here for the first time we show that the antiproliferative effect of CPT is associated with inhibition of the signaling pathway of mTOR, a master kinase that regulates cell proliferation (5, 13), suggesting that CPT may be a new mTOR inhibitor. Further understanding of the underlying mechanism may lead to the design of novel tumor-selective therapeutics. Recent studies have shown that CPT inhibits prostate cancer cell growth by inhibiting the phosphorylation of Stat3 through a Janus-activated kinase-2-independent mechanism (36). It has been suggested that Stat3 is positively regulated by mTOR (46). It would be interesting to elucidate whether CPT downregulation of Stat3 phosphorylation is through inhibition of mTOR signaling.

We found that CPT inhibited the proliferation of Rh30 and DU145 by arresting cells in G₁-G₀ phase of the cell cycle. This is an interesting finding because both Rh30 and DU145 cells express mutant *p53* alleles, losing the function

of *p53* (37, 47). Therefore, it seems that CPT is able to arrest cells in the G₁-G₀ phase in a *p53*-independent manner. Mutations of *p53* have been documented in more than 50% of human tumors (48). Our findings suggest that CPT may have potential applications as a chemotherapeutic agent against *p53*-mutant tumor cells, which are resistant to irradiation therapy or other chemotherapies. However, we also noticed that CPT inhibits tumor cell proliferation at rather high concentrations, with IC₅₀ values of 3 to 5 μmol/L in Rh30 and DU145 cells. Animal studies have revealed that achievable maximal plasma concentrations of CPT were only 14.7 to 55.8 ng/mL (i.e., 0.05–0.18 μmol/L) in rats and 3.1 to 227.4 ng/mL (i.e., 0.01–0.77 μmol/L) in dogs after oral administration of a single dose (30–180 mg/kg for rats; 17.8–1,080 mg/kg for dogs) of CPT (49). Therefore, it is necessary to develop a new formula (such as nanoparticles) of CPT to increase its bioavailability or more potent CPT analogues for cancer prevention and treatment.

Eukaryotic cell cycle progression is balanced by cyclins/CDKs and CDK inhibitors (1). Early G₁ transition is mainly regulated by cyclin D complexed with CDK4 and/or CDK6, whereas late G₁-S and early S-phase transitions are regulated by cyclin E coupled with CDK2 (1, 3, 4). To investigate how CPT arrests cells in G₁-G₀ phase, we examined the effects of CPT on the expression of cell cycle regulatory proteins. Our Western blot analysis consistently revealed that CPT downregulated the protein expression of cyclin D1 but failed to alter the expression of cyclin A, cyclin B1, cyclin E, and CDK2 in all cell lines tested, including Rh30 (Fig. 3), DU145, and MCF-7 (data not shown). Our results further suggest that CPT downregulation of cyclin D1 expression is due to inhibition of mTOR signaling. This is supported by the findings that overexpression of constitutively active mTOR in Rh30 cells conferred high

resistance to CPT inhibition of cyclin D1 expression (Fig. 5). Our data are in agreement with previous findings that mTOR controls the synthesis of cyclin D1 (42). Taken together, the results suggest that CPT inhibition of mTOR-mediated expression of cyclin D1 may be primarily responsible for G₁-G₀ cell cycle arrest.

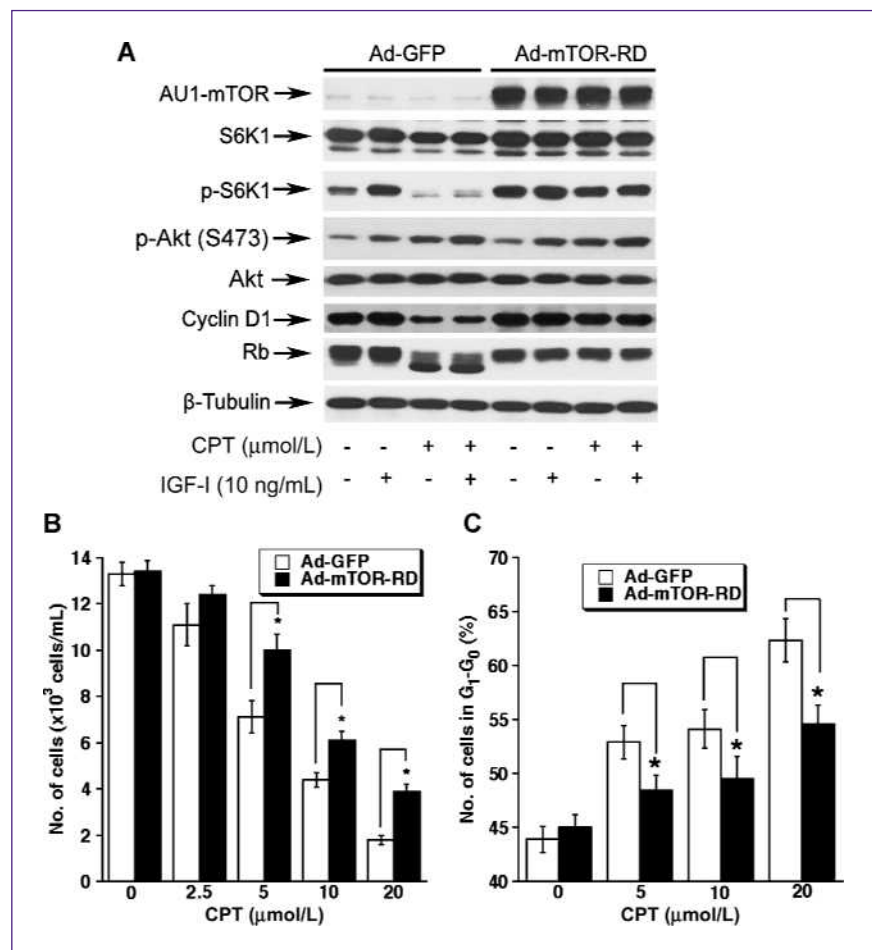
In the present study, we found that CPT inhibited mTORC1-mediated phosphorylation of S6K1 and 4E-BP1, but increased mTORC2-mediated phosphorylation of Akt. It has been described that S6K1 phosphorylates insulin receptor substrate 1, promoting its degradation (50). Inhibition of S6K1 activity prevents the phosphorylation of insulin receptor substrate 1 (S636/639), resulting in accumulation of insulin receptor substrate 1 and activation of its downstream kinases, such as PI3K and Akt, by a feedback regulating mechanism (50–53). Our preliminary data indicate that CPT did not alter either the protein expression of PI3K (p110 and p85) or the phosphorylation of p85 (data not shown). Whether CPT activates Akt through this feedback regulating mechanism remains to be determined. Undoubtedly, it is of greater importance to elucidate how CPT inhibits mTORC1 signaling, as this may provide direct evidence

for the development of more potent new CPT analogues for cancer prevention and treatment.

The effects of CPT on CDK inhibitors were complex and seemed to be cell line dependent. In Rh30, CPT upregulated the expression of p21^{Cip1} but downregulated the expression of p27^{Kip1}; in MCF-7 cells, CPT downregulated the expression of p21^{Cip1} but upregulated the expression of p27^{Kip1}; in DU145 cells, CPT downregulated the expression of both p21^{Cip1} and p27^{Kip1} (data not shown). Both Rh30 and DU145 cells express mutant *p53* alleles whereas MCF-7 cells express wild-type *p53* (37, 48), suggesting that CPT upregulation or downregulation of p21^{Cip1} was independent of *p53*. It has been described that inhibition of mTOR results in accumulation of p27^{Kip1} (54) and reduction of p21^{Cip1} expression (55). Further studies may help unveil whether upregulation of p27^{Kip1} and downregulation of p21^{Cip1} expression in MCF-7 or DU145 cells are a consequence of mTOR inhibition.

The Rb tumor-suppressor protein, a major target of CDKs, plays a pivotal role in the regulation of cell cycle progression from G₁ to S phase (2). The activity of Rb is controlled by its phosphorylation status (2). Hypophosphorylated Rb is the active growth-inhibitory form, which

Fig. 6. Expression of constitutively active mTOR confers high resistance to CPT inhibition of mTOR signaling, cyclin D1 expression, and Rb phosphorylation and partially prevents CPT inhibition of cell proliferation in cancer cells. **A**, Rh30 cells, grown in six-well plates and infected with Ad-GFP or Ad-mTOR-RD for 24 h, were treated with or without CPT (10 μ mol/L) for 2 h, followed by stimulation with or without IGF-I (10 ng/mL) for 1 h. The cell lysates were subjected to Western blot analysis with the indicated antibodies. β -Tubulin was used as a loading control. **B** and **C**, Rh30 cells, grown in six-well plates and infected with Ad-GFP or Ad-mTOR-RD for 24 h, were treated with CPT (0–20 μ mol/L) for 24 h, followed by cell counting using a Beckman Coulter Counter (B) or cell cycle analysis (C). Columns, mean ($n = 3$); bars, SE. *, $P < 0.05$, versus control.



binds E2F and prevents G₁-S transition; in contrast, hyperphosphorylated Rb is released from E2F and is the inactive form (2). The D cyclins and their counterpart CDKs have been suggested to be the most important regulators of Rb phosphorylation (2, 56). Here, we observed a profound loss of Rb phosphorylation after 24 hours of exposure to CPT in Rh30 cells. Overexpression of constitutively active mTOR rendered high resistance to CPT inhibition of cyclin D1 expression and Rb phosphorylation, further supporting the notion that CPT-induced G₁-G₀ cell cycle arrest is a consequence of suppression of mTOR-mediated cyclin D1 expression and Rb phosphorylation.

In summary, we have shown that CPT inhibits the proliferation of tumor cells by arresting the cells in G₁-G₀ cell cycle, which is related to downregulation of cyclin D1 expression, resulting in hypophosphorylation of Rb. The antiproliferative effect of CPT is probably linked to inhibition of the mTOR signaling pathway.

References

- Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* 2009;9:153–66.
- Burkhardt DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer* 2008;8:671–82.
- Santamaria D, Ortega S. Cyclins and CDKs in development and cancer: lessons from genetically modified mice. *Front Biosci* 2006;11:1164–88.
- Lindqvist A, Rodríguez-Bravo V, Medema RH. The decision to enter mitosis: feedback and redundancy in the mitotic entry network. *J Cell Biol* 2009;185:193–202.
- Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell* 2007;12:9–22.
- Fonseca BD, Smith EM, Lee VH, Mackintosh C, Proud CG. PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signaling downstream of this complex. *J Biol Chem* 2007;282:24514–24.
- Hara K, Maruki Y, Long X, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* 2002;110:177–89.
- Kim DH, Sarbassov DD, Ali SM, et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 2002;110:163–75.
- Kim DH, Sarbassov DD, Ali SM, et al. GβL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell* 2003;11:895–904.
- Loewith R, Jacinto E, Wullschleger S, et al. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* 2002;10:457–68.
- Sancak Y, Thoreen CC, Peterson TR, et al. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell* 2007;25:903–15.
- Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol* 2007;9:316–23.
- Fasolo A, Sessa C. mTOR inhibitors in the treatment of cancer. *Expert Opin Investig Drugs* 2008;17:1717–34.
- Frias MA, Thoreen CC, Jaffe JD, et al. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr Biol* 2006;16:1865–70.
- Jacinto E, Loewith R, Schmitt A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* 2004;6:1122–8.
- Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 2004;14:1296–302.
- Jacinto E, Facchinetti V, Liu D, et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* 2006;127:125–37.
- Pearce LR, Huang X, Boudeau J, et al. Identification of Protor as a novel Rictor-binding component of mTOR complex-2. *Biochem J* 2007;405:513–22.
- Yang Q, Inoki K, Ikenoue T, Guan KL. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev* 2006;20:2820–32.
- Woo SY, Kim DH, Jun CB, et al. PRR5, a novel component of mTOR complex 2, regulates platelet-derived growth factor receptor β expression and signaling. *J Biol Chem* 2007;282:25604–12.
- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005;307:1098–101.
- Dada S, Demartines N, Dormond O. mTORC2 regulates PGE2-mediated endothelial cell survival and migration. *Biochem Biophys Res Commun* 2008;372:875–9.
- Facchinetti V, Ouyang W, Wei H, et al. The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J* 2008;27:1932–43.
- Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J* 2008;27:1919–31.
- Cheng TO. Cardiovascular effects of Danshen. *Int J Cardiol* 2007;121:9–22.
- Zhou L, Zuo Z, Chow MS. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J Clin Pharmacol* 2005;45:1345–59.
- Wojcikowski K, Johnson DW, Gobe G. Herbs or natural substances as complementary therapies for chronic kidney disease: ideas for future studies. *J Lab Clin Med* 2006;147:160–6.
- Wang BE. Treatment of chronic liver diseases with traditional Chinese medicine. *J Gastroenterol Hepatol* 2000;15 Suppl:E67–70.
- Yu XY, Lin SG, Chen X, et al. Transport of cryptotanshinone, a major active triterpenoid in *Salvia miltiorrhiza* Bunge widely used in the treatment of stroke and Alzheimer's disease, across the blood-brain barrier. *Curr Drug Metab* 2007;8:365–78.
- Jin DZ, Yin LL, Ji XQ, Zhu XZ. Cryptotanshinone inhibits cyclooxygenase-2 enzyme activity but not its expression. *Eur J Pharmacol* 2006;549:166–72.
- Suh SJ, Jin UH, Choi HJ, et al. Cryptotanshinone from *Salvia miltiorrhiza* BUNGE has an inhibitory effect on TNF-α-induced matrix metalloproteinase-9 production and HASMC migration via

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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- down-regulated NF- κ B and AP-1. *Biochem Pharmacol* 2006;72:1680–9.
32. Kim EJ, Jung SN, Son KH, et al. Antidiabetes and antiobesity effect of cryptotanshinone via activation of AMP-activated protein kinase. *Mol Pharmacol* 2007;72:62–72.
 33. Don MJ, Liao JF, Lin LY, Chiou WF. Cryptotanshinone inhibits chemotactic migration in macrophages through negative regulation of the PI3K signaling pathway. *Br J Pharmacol* 2007;151:638–46.
 34. Zhang F, Zheng W, Pi R, et al. Cryptotanshinone protects primary rat cortical neurons from glutamate-induced neurotoxicity via the activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. *Exp Brain Res* 2008;193:109–18.
 35. Nizamutdinova IT, Lee GW, Son KH, et al. Tanshinone I effectively induces apoptosis in estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) breast cancer cells. *Int J Oncol* 2008;33:485–91.
 36. Shin DS, Kim HN, Shin KD, et al. Cryptotanshinone inhibits constitutive signal transducer and activator of transcription 3 function through blocking the dimerization in DU145 prostate cancer cells. *Cancer Res* 2009;69:193–202.
 37. Beevers CS, Li F, Liu L, Huang S. Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells. *Int J Cancer* 2006;119:757–64.
 38. Coward P, Wada HG, Falk MS, et al. Controlling signaling with a specifically designed G α -coupled receptor. *Proc Natl Acad Sci U S A* 1998;95:352–7.
 39. Briaud I, Dickson LM, Lingohr MK, McCuaig JF, Lawrence JC, Rhodes CJ. Insulin receptor substrate-2 proteasomal degradation mediated by a mammalian target of rapamycin (mTOR)-induced negative feedback down-regulates protein kinase B-mediated signaling pathway in β -cells. *J Biol Chem* 2005;280:2282–93.
 40. Liu L, Chen L, Chung J, Huang S. Rapamycin inhibits F-actin reorganization and phosphorylation of focal adhesion proteins. *Oncogene* 2008;27:4998–5010.
 41. Liu L, Li F, Cardelli JA, Martin KA, Blenis J, Huang S. Rapamycin inhibits cell motility by suppression of mTOR-mediated S6K1 and 4E-BP1 pathways. *Oncogene* 2006;25:7029–40.
 42. Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L, Ferrari S. Rapamycin inhibition of the G $_1$ to S transition is mediated by effects on cyclin D1 mRNA and protein stability. *J Biol Chem* 1998;273:14424–29.
 43. Chen Y, Knudsen ES, Wang JY. The RB/p107/p130 phosphorylation pathway is not inhibited in rapamycin-induced G $_1$ -prolongation of NIH3T3 cells. *Oncogene* 1996;13:1765–71.
 44. Brunn GJ, Hudson CC, Sekuliac A, et al. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* 1997;277:99–101.
 45. Holz MK, Blenis J. Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. *J Biol Chem* 2005;280:26089–93.
 46. Zhou J, Wulffkuhle J, Zhang H, et al. Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. *Proc Natl Acad Sci U S A* 2007;104:16158–63.
 47. Isaacs WB, Carter BS, Ewing CM. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. *Cancer Res* 1991;51:4716–20.
 48. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323–31.
 49. Pan Y, Bi HC, Zhong GP, et al. Pharmacokinetic characterization of hydroxypropyl- β -cyclodextrin-included complex of cryptotanshinone, an investigational cardiovascular drug purified from Danshen (*Salvia miltiorrhiza*). *Xenobiotica* 2008;38:382–98.
 50. Tremblay F, Marette A. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J Biol Chem* 2001;276:38052–60.
 51. Tzatsos A, Kandror KV. Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. *Mol Cell Biol* 2006;26:63–76.
 52. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006;66:1500–8.
 53. Wan X, Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene* 2007;26:1932–40.
 54. Nourse J, Firpo E, Flanagan WM, et al. Interleukin-2-mediated elimination of the p27^{Kip1} cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* 1994;372:570–73.
 55. Beuvink I, Boulay A, Fumagalli S, et al. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. *Cell* 2005;120:747–59.
 56. Polager S, Ginsberg D. E2F—at the crossroads of life and death. *Trends Cell Biol* 2008;18:528–35.

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