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Cryptotanshinone, a Stat3 inhibitor, suppresses colorectal cancer proliferation and growth in vitro

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Abstract Cryptotanshinone (CPT) is a natural compound extracted from herbal medicine that has been previously shown to possess antitumor properties in various types of human cancer cells. In the present study, we examined the potential role of CPT in the treatment of colorectal cancer. Using SW480, HCT116, and LOVO colorectal cancer cell lines, the effects of CPT on cell viability, apoptosis, and tumorigenicity were evaluated. The results showed that CPT significantly inhibited the growth and viability of SW480, HCT116, and LOVO cell lines by inducing apoptosis and prevented anchorage dependent growth on agar. In addition, CPT inhibited the activation of Signal transducer and activator of transcription 3 (Stat3) pathways in colorectal cancer cells. Stat3 is a transcription factor that mediates the expression of various genes associated with many cellular processes, such as inflammation and cell growth, and has been shown to promote several cancer types, including colorectal cancer. These findings indicate that CPT may be a potential candidate for the treatment and prevention of colorectal cancer in part by inhibiting the activation of Stat3.

Keywords Cryptotanshinone · Colorectal cancer treatment · Signal transducer and activator of transcription 3 · Proliferation · Apoptosis

Introduction

Colorectal cancer is the third most common cancer worldwide and the fourth most common cause of cancer-related mortality [1]. Despite the availability of screening test, approximately more than half of the colorectal cancer cases are diagnosed at late stages when treatment is more difficult [2]. Chemotherapy is the primary intervention for the treatment of colorectal cancer; however, the use of chemotherapy is limited due to its side effects, which significantly impact the patient quality of life. Consequently, many efforts are being made to find alternative agents with fewer side effects to complement conventional chemotherapy. The use of herbal medicine derived from traditional Chinese medicine is an alternative approach for the prevention and treatment of cancer with fewer side effects.

STAT (Signal Transducer and Activator of Transcription) proteins regulate many aspects of growth, survival, and differentiation in cells. There are seven mammalian Stat family members that have been identified: Stat1, Stat2, Stat3, Stat4, Stat5 (Stat5α and Stat5β), and Stat6. Stat3 appears to be more generally transcribed than the other members, and recent data demonstrate its role in a wide variety of physiological processes. Stat3 has been implicated in cancer, following the discovery

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that Stat3 is required for malignant transformation in v-SRC-transformed cells [3]. Activated Stat3 has been observed in malignant cells and is capable of inducing the expression of a large number of genes involved in tumorigenesis, thus becoming a suitable target for preventing and treating cancer [4–6]. In the case of colorectal cancer, Stat3 activation is associated with adverse clinical outcome [7].

Cryptotanshinone (CPT) is a quinoid diterpene purified from the root of medicinal plant Danshen (*Salvia miltiorrhiza*) that has been widely used in the clinic for various diseases, including cardiac fibrosis [8], acute lung injury [9], and arthritis [10]. CPT has been previously shown to inhibit growth and induce apoptosis in a number of human cancer cell lines [11]. In prostate cancer cells, CPT induced cell apoptosis by suppressing mammalian target of rapamycin (mTOR)-mediated cyclin D1 expression [12] and inhibiting Stat3 activity [13]. In myeloid leukemia cells, CPT induced apoptosis by inhibiting the eukaryotic initiation factor 4E (eIF4E) regulatory system [14] and through ROS-dependent activation of caspase-8 and p38 MAPK [15]. While the anticancer activities of CPT against diverse types of cancer cells have been reported, the efficacy and molecular mechanisms of CPT remain unclear in colorectal cancer cells.

The current study investigated the efficacy of CPT against human colorectal cancer in vitro. Previous research has suggested antitumor effects of CPT and its ability to inhibit Stat3 activation. We evaluated the effect of CPT on human colorectal cancer cell viability, cell apoptosis, tumorigenicity, and Stat3 signaling.

Materials and methods

Reagents

CPT (purity $\geq$ 99 % purity by high-performance liquid chromatography, with chemical structure as shown in Fig. 1a) was supplied by National Food and Drug Control Institute, China. CPT was dissolved in DMSO to prepare a 50 mM stock solution (with a final DMSO concentration $\leq$0.1 %) and stored in -20 °C. RPMI-1640, Dulbecco's Modified Eagle's Medium (DMEM), and 0.25 % Trypsin–EDTA were purchased from Gibco (Life Technologies, Grand Island, NY, USA). Fetal Bovine Serum (FBS) was purchased from Atlantic Biological. TACS MTT Cell Proliferation Assay kits were ordered from Trevigen (Helgerman, Gaithersburg, MD, USA). RIPA buffer was purchased from Thermo Scientific (Thermo scientific, Rockford, IL, USA). Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (San Diego, CA, USA).

Antibodies

p-Stat3^{Tyr705}, Stat3, Cyclin D1, Survivin, cleaved-caspase-3, Bcl-xl, Bcl-2, p21, p27, p-EGFR, EGFR, and goat-anti-rabbit and anti-mouse horseradish peroxidase (HRP) conjugates were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin was ordered from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

HCT116, SW480, and LOVO colon cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository (National Cancer Institute, USA). HCT116 and LOVO cells were cultured in RPMI1640 supplemented with 10 % FBS, 1 % penicillin streptomycin, and 1 % L-Glutamine. SW480 cells were cultured in DMEM supplemented with 10 % FBS, 1 % penicillin streptomycin, and 1 % L-Glutamine. Cultures were maintained in a humidified incubator at 37 °C in 5 % CO_2.

Cell viability assay

Cells were seeded at a density of 2×10^4 cells per well in a flat-bottomed 96-well plate and cultured for 24 h. Cells were treated with CPT (5, 10, 20, 40, 80, and 100 μM) or 0.1 % DMSO for 24, 48, and 72 h. Subsequently, 10 μl MTT reagent was added into each well and incubated for another 4 h until purple dye is visible. 100 μl of detergent reagent was added to the plate and was incubated at room temperature in the dark for 2–4 h. Absorbance was measured at 570 nm with BIO-RAD Model 680 Microplate reader according to the instruction of the TACS MTT Cell Proliferation Assay kits (Trevigen, Helgerman, Gaithersburg, MD, USA). MTT Cell Proliferation Assay kit measures viable cells that have active NAD(P)H-dependent cellular oxidoreductase activity and not dead or quiescence cells. The relative cell viability was expressed as the ratio of the optical density of CPT-treated cells to that of DMSO control cells. The experiments were independently performed at least twice, each in triplicate.

Cell apoptosis assay

HCT116 and SW480 cells were seeded in 100-mm dishes at a density of 2×10^6 cells per dish in growth medium and grown overnight at 37 °C in a humidified incubator with 5 % CO_2. Cells were treated with CPT (5, 10, 25, 50 μM) or 0.1 % DMSO for 24 h. Apoptosis was determined with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA). The experiments were independently performed at least twice, each in triplicate.

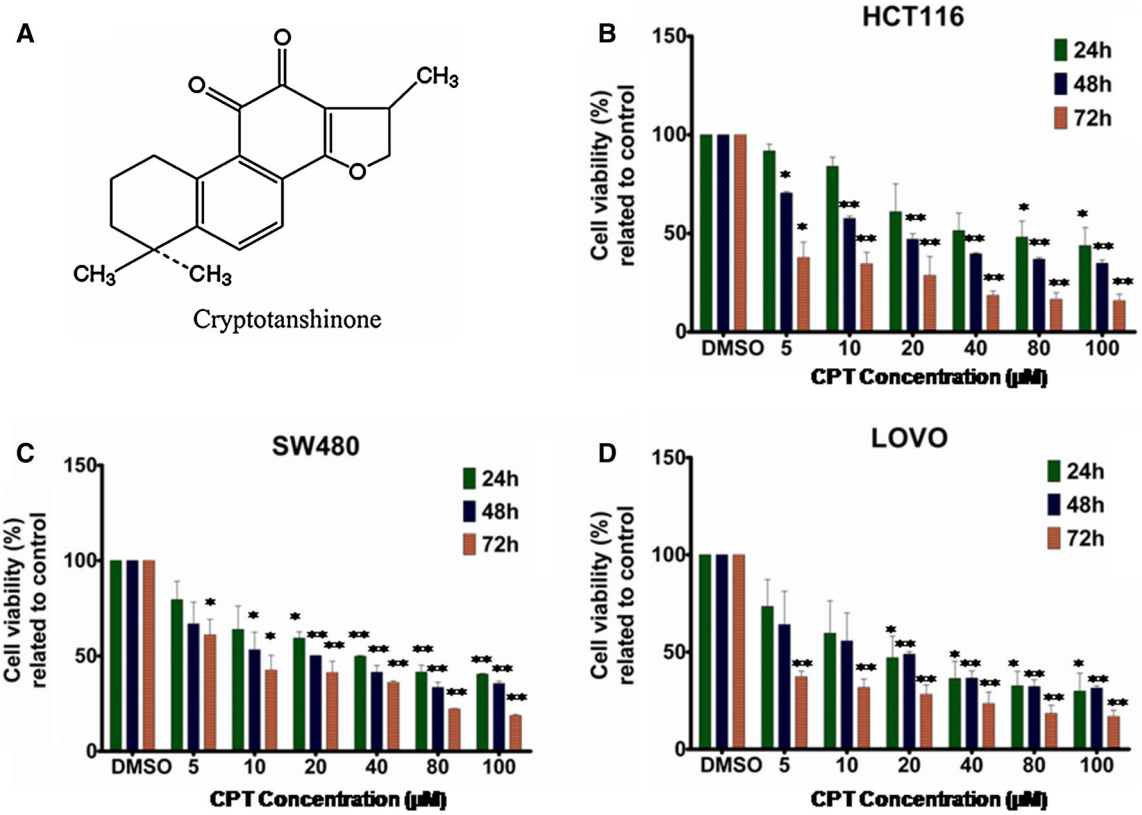

Fig. 1 Cryptotanshinone inhibits colorectal cell viability. **a** Chemical structure of Cryptotanshinone. 2×10^4 cells **b** HCT1116, **c** SW480, **d** LOVO were seeded into 96-well plates and after 24 h were treated with 5–100 μmol/L CPT or 0.1 % DMSO. At 24, 48, and 72 h after CPT treatment, the cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in "Materials and methods" section. Absorbance at 570 nm was detected with Microplate reader. The experiments were independently performed at least twice, each in triplicate. $*p < 0.05$ and $**p < 0.01$ indicate a significant difference compared with DMSO control by *t* test. *CPT* Cryptotanshinone

Soft agar assay

HCT116 cells and SW480 cells were trypsinized and re-suspended at 6000 cells in 2x RPMI media. Cells were treated with 0.1 % DMSO or CPT (5, 10, 25, and 50 μM). The bottom layer consisted of 2 ml of 1.2 % agarose. The cell suspensions were mixed 1:1 with 0.5 % agarose (2 ml/well for a 6 well plate) and layered on top. The cells were maintained in an incubator for 10 days after which the colonies were stained with Nitrotetrazolium Blue chloride (NBT), scanned, and counted with GelCount (Oxford Optronix Ltd, Oxford United Kingdom). The experiments were independently performed at least twice, each in triplicate.

Western blotting

Colon cancer cells were seeded 2×10^4 on 100-mm plates and treated with either DMSO vehicle (0.1 %) or various concentrations of CPT (5, 10, 25, and 50 μM) for 24 h or 25 μM CPT for various times (0, 0.5, 1, 4, 8, 12,24, and 36 h). The whole-cell protein extracts were isolated using RIPA lysis buffer (Thermo scientific, Rockford, IL, USA) containing protein inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was determined by Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Approximately 30 μg total protein was loaded and fractionated by SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad), and probed with primary antibodies: p-Stat3^{Tyr705}, Stat3, Cyclin D1, Survivin, cleaved-caspase-3, Bcl-xl, Bcl-2, p21, p27, and p-EGFR, EGFR. Signal was detected using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) and quantified by optical densitometry with Image J software. The experiments were independently performed at least twice, each in triplicate.

Statistical analyses

All data are presented as the mean ± standard deviation. The significance of the difference between groups was evaluated by one-way repeated-measures analysis of

variance (ANOVA) or Student's t test, and multiple comparisons with Prism 5.0 software. $P < 0.05$ was considered to be statistically significant.

Results

CPT inhibits colorectal cancer cell viability

To study the effect of CPT on cell viability in HCT116, SW480, and LOVO colorectal cancer cell lines, MTT assay was performed. Cells were treated with various concentrations of CPT (5, 10, 20, 40, 80, and 100 μM) for 24, 48, and 72 h. CPT substantially reduced the cell viability of HCT116, SW480 and LOVO colorectal cancer cell lines in a dose- and time-dependent manner with an IC50 of 11, 12, and 9 μM, respectively (Fig. 1b–d) after 72 h. CPT treatment had no effect on the cell viability of non-tumor human embryonic kidney HEK293 cells (Supplementary Fig. S1). These results show that CPT exerts cytotoxic effects on HCT116, SW480, and LOVO colorectal cell lines, but not on non-tumor cells. Based on the chemosensitivity MTT assay, further analysis was limited to HCT116 and SW480 cell lines, which displayed a similar IC50 and excluded the exquisitely sensitive LOVO cell line.

CPT induces apoptosis in colorectal cancer cells

CPT treatment significantly attenuated the cell viability of HCT116 and SW480 cells. To determine if the effect of CPT on cell viability is mediated by apoptosis, FITC Annexin V apoptosis detection assay was performed. Consistently, apoptosis analysis showed an increased accumulation in the number of apoptotic cells in a dose-dependent manner in both HCT116 and SW480 cell lines after being treated with CPT for 24 h (Fig. 2a, b). Treatment with 25 μM of CPT resulted in a 48 and 26 % increase in apoptotic cells in HCT116 and SW480 cells, respectively ($p < 0.05$). To confirm these results, the activation of caspase-3, a key molecule in the intrinsic apoptosis pathway, was evaluated by Western blot. As expected, CPT induced the cleavage of caspase-3 in a dose-dependent manner (Fig. 2c, d). Bcl-2 is a

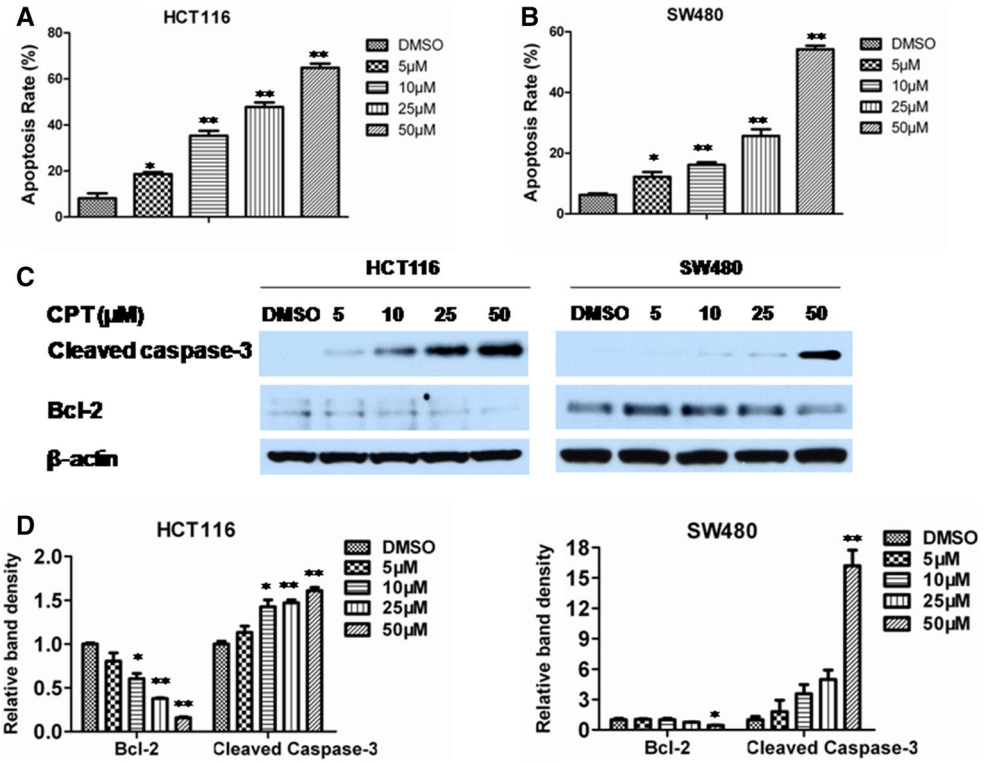

Fig. 2 Cryptotanshinone induces colorectal cell apoptosis. **a** HCT116 cells or **b** SW480 cells were grown in 100-mm dishes (2×10^6 cells per dish) and treated with indicated concentration of Cryptotanshinone (5-50 μmol/L) for 24 h. Cells were stained with Annexin V-FITC and Propidium Iodide, followed by flow cytometric analysis. Results are shown as mean ± SE ($n = 3$). The experiments were independently performed at least twice, each in triplicate. $*p < 0.05$ and $**p < 0.01$ indicate a significantly different compared with DMSO control. **c** CPT activated cleavage of caspase-3 and inhibited Bcl-2. HCT116 cells or SW480 cells were treated with 5–50 μmol/L CPT for 24 h. Cleaved caspase-3 and Bcl-2 expression were analyzed by Western blot. Blots are representatives of three independent experiments. **d** Densitometry analyses of Western blots were determined by Image J to quantify the relative protein levels of Bcl-2 versus β-actin and Cleaved Caspase-3 versus β-actin. Three independent experiments were analyzed. $*p < 0.05$ and $**p < 0.01$ indicate a significant difference compared with DMSO control by *t* test

key anti-apoptosis regulator that is often over-expressed in human cancers and can result in chemotherapy resistance [16–18]. Our results show that CPT inhibited Bcl-2 level in a dose-dependent manner (Fig. 2c, d). Taken together, these results suggest that the effect of CPT on colorectal cancer cell viability is at least in part mediated by apoptosis.

CPT inhibits Stat3 activation in colorectal cancer cells

We show that CPT can induce apoptosis in HCT116 and SW480 cells. Previous reports show that CPT can inhibit Stat3 activity in other cell types [13, 19], and Stat3 is known to regulate gene products associated with cell cycle progression [20–22]. To determine if CPT can inhibit Stat3 activation in colorectal cancer cell lines, we evaluated Stat3 phosphorylation by Western blot. HCT116 and SW480 cells were treated with various concentrations of CPT (5, 10, 25, and 50 μM) for 24 h and treated with 25 μM CPT for various times (0, 0.5, 1, 4, 8, 12, 24, and 36 h). CPT significantly inhibited the phosphorylation of Stat3 in a dose- and time-dependent manner in both HCT116 and SW480 cell lines (Fig. 3). The inhibition of Stat3 was achieved at a much lower concentration and significantly shorter time in HCT116 cells compared to SW480 cells (Fig. 3a, b). In addition, CPT inhibited phosphorylation of EGFR (Epidermal growth factor receptor), an upstream regulator of Stat3 activation [23], in HCT116 and SW480 cell lines (Fig. 4a, b); however, EGFR inactivation was only observed at higher concentrations in both the HCT116 and SW480 cell lines. Thus, STAT3 inhibition by CPT is likely not mediated by changes in EGRF activity.

CPT inhibits CyclinD1 and survivin in colorectal cancer cells

Stat3 activity has been implicated in promoting various types of human cancers by regulating gene products involved in cell survival and proliferation [24, 25]. CyclinD1 and Survivin are two well-known examples of Stat-related survival gene products [26, 27]. CyclinD1 is a major cell cycle regulatory protein that functions as a cofactor for several transcription factors [28] and its overexpression has been linked to the development and progression of cancer [29]. Survivin is a negative regulator of apoptosis that can inhibit the activation of caspases to promote cellular survival under otherwise apoptotic conditions [30]. Our results

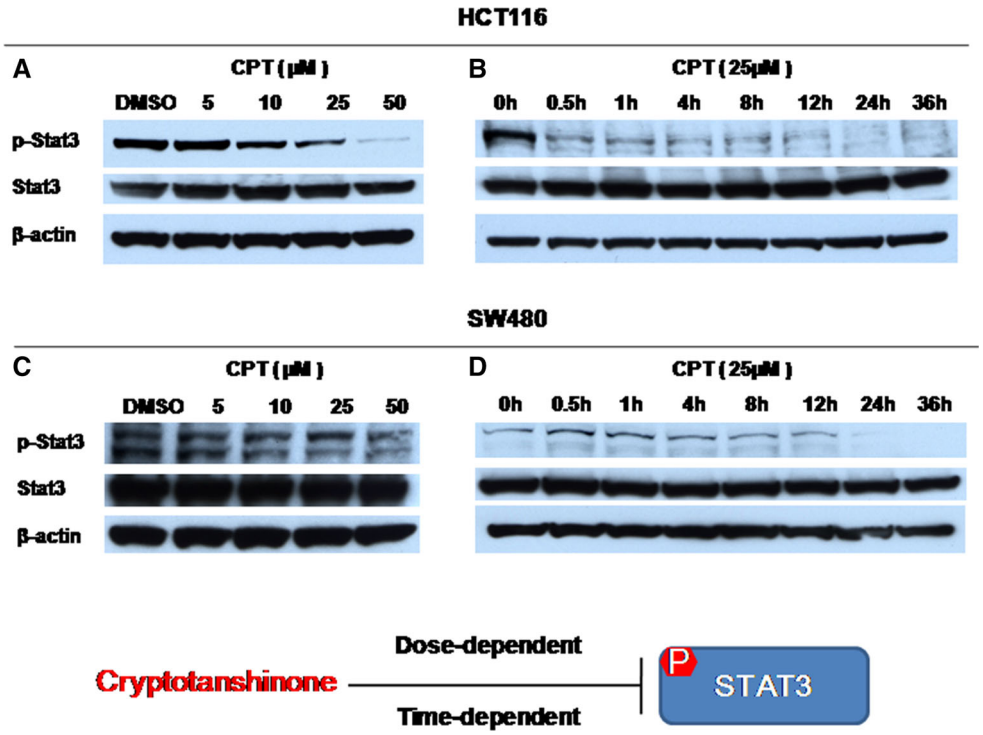

Fig. 3 Cryptotanshinone suppresses Stat3 phosphorylation in colorectal cancer cells. HCT116 and SW480 cells were seeded as 2×10^4 in 100-mm plates and after 24 h were treated with 0.1 % DMSO, 5, 10, 25, or 50 μmol/L CPT. After 24 h of treatment, extracts were analyzed by Western blotting analysis. **a**, **c** Total and phosphorylated Stat3. Representative blots of two colon cancer cell lines HCT116 (a) SW480 (**c**). Blots are representative of three independent experiments. **b**, **d** Inhibition of Stat3 by CPT treatment. Time course inhibition of Stat3 **b** and **d** in HCT1116 and SW480 cells treated with 25 μmol/L CPT from 0 to 36 h and analyzed by Western blot. Phospho-Stat3 (p-Stat3). Blots are representative of three independent experiments

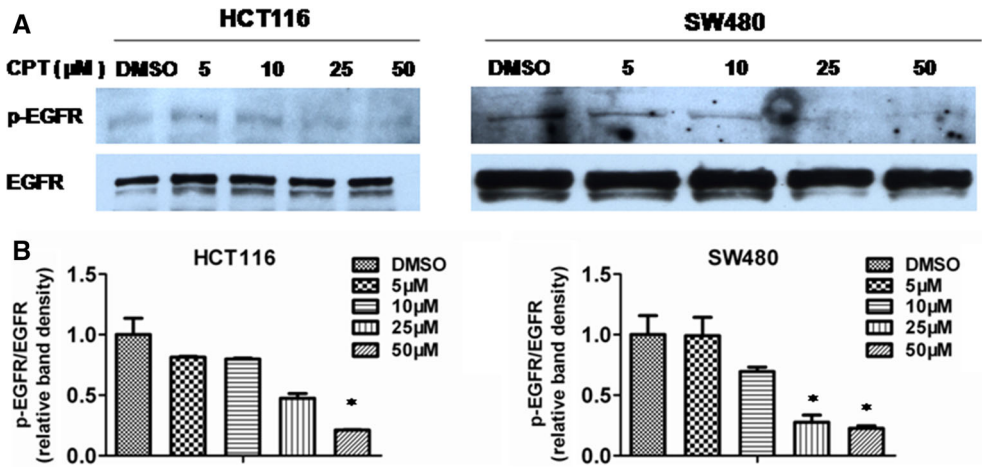

Fig. 4 Cryptotanshinone inhibits phosphorylation of EGFR in colorectal cancer cells. HCT116 and SW480 cells were seeded as 2×10^4 in 100-mm plates and after 24 h were treated with 0.1 % DMSO, 5, 10, 25, or 50 μmol/L CPT. After 24 h of treatment, extracts were analyzed by Western blotting analysis. **a** Total and phosphorylated EGFR representative blots of two colon cancer cell lines HCT116 and SW480. Blots are representative of three independent experiments. **b** Densitometry analyses of Western blots were determined by Image J to quantify the relative protein levels of p-EGFR versus EGFR. Three independent experiments were analyzed. $*p < 0.05$ indicates a significant difference compared with DMSO control by *t* test

show that treating HCT116 and SW480 cells with CPT significantly attenuated the expression of both CyclinD1 and Survivin in a dose-dependent and time-dependent manner (Fig. 5).

CPT induces p21 and p27 expression in colorectal cancer cells

The cyclin-dependent kinase inhibitor p21 (also known as p21WAF1/Cip1) promotes cell cycle arrest in response to many stimuli. It can function as both a sensor and an effector of multiple anti-proliferative signals [31]. In addition to inhibiting Stat-related survival gene products, we also show that CPT can induce p21 expression in a dose- and time-dependent manner in both HCT116 and SW480 cells (Fig. 5). CPT can also induce p27 expression in colorectal cancer cells (Fig. 5e), which is frequently down-regulated in cancer and correlates with poor prognosis [32, 33].

CPT inhibits colorectal cancer cell oncogenic phenotypes

To evaluate the effects of CPT on tumor phenotypes of CRC cells, soft agar assay was performed. Soft agar assays are commonly used to evaluate the efficacy and sensitivity of chemotherapeutic agents by monitoring anchorage-independent three-dimensional colony formation in a semi-solid culture medium. This method delivers results that are comparable to those obtained when injecting tumorigenic cells into nude mice [34]. Following pre-treatment with CPT, colorectal cancer cells, HCT116 or SW480 cell lines, were placed in soft agar and colonies were counted after 10 days. As shown in Fig. 6, treatment with 5, 10, 25, and 50 μM of CPT provided significant concentration-dependent inhibition of colony formation by 79, 84, 90, and 95 percent, respectively, ($p < 0.05$) compared to control. These results show that CPT affected the ability of colorectal cancer cells to form colonies in soft agar, which may be contributed to the inhibition of the Stat3 pathway.

Discussion

The use of Chinese herbal medicines and natural products has received considerable attention in recent years for the prevention and treatment of many health conditions. CPT is one of the active constituents extracted from the dry root and rhizome of medicinal plant *Salvia miltiorrhiza* (Chin. Danshen) and has been used clinically in China for centuries as an anticoagulant for ischemic syndromes and for the prevention and treatment of Alzheimer's disease [35]. More recently, CPT has been shown to possess anticancer properties in human prostate, leukemic, melanoma, and glioma cancer cells [11, 19, 36–38]. The efficacy and mechanism of CPT in colon cancer cells has yet to be investigated. In the present study, we investigated the anticancer activity of CPT in colorectal cancer. The concentration of CPT used in the current study was based on that of a previous study in mice where a 25 mg/kg dose of CPT (human equivalent dose of 2.34 mg/kg) was used and found to be well tolerated with no reported toxicity [39]. In

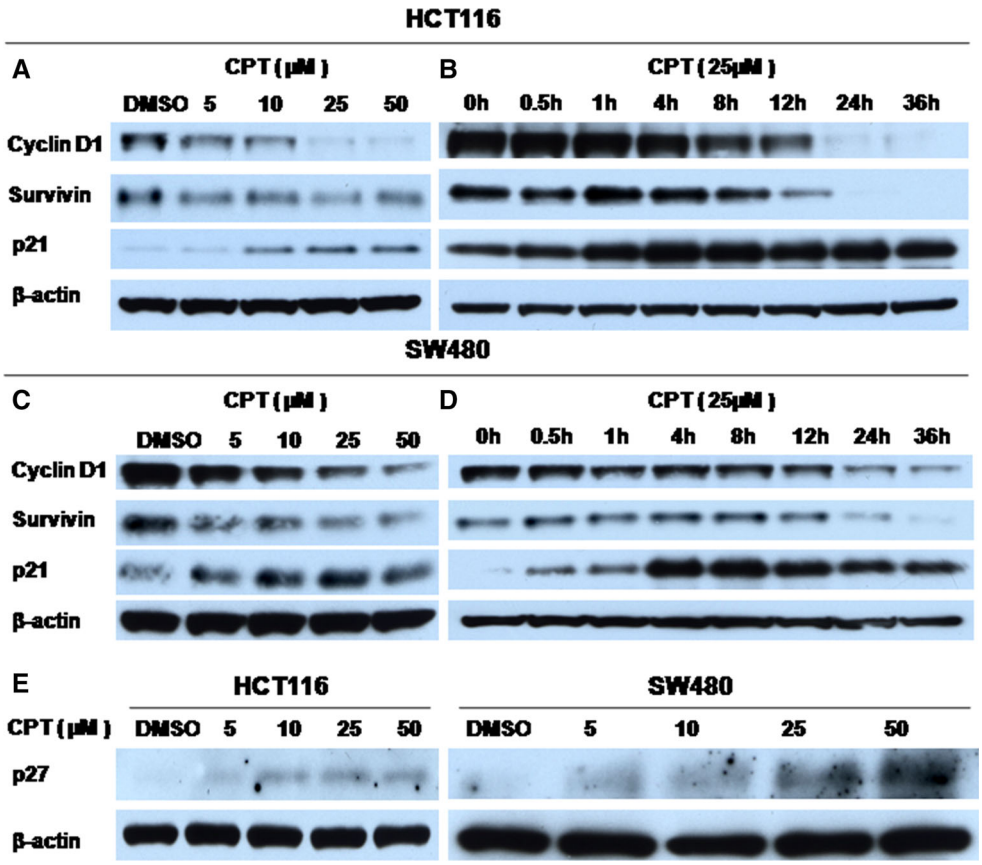

Fig. 5 Cryptotanshinone inhibits CyclinD1 and Survivin and induces p21and p27 in colorectal cancer cells. Expression levels of candidate Stat3 targets, Cyclin D1 and Survivin, were inhibited by CPT. **a**, **c** HCT116 cells or SW480 cells were treated with 0.1 % DMSO or 5–50 μmol/L CPT for 24 h. Cyclin D1, Survivin expression was analyzed by Western blot. Blots are representatives of three independent experiments. Time course inhibition of Cyclin D1 and Survivin in HCT1116 cells (**b**) or SW480 cells (**d**) treated with 25 μmol/L CPT from 0 to 36 h and analyzed by Western blot. Phospho-Stat3 (p-Stat3). Blots are representative of three independent experiments. CPT induces p21 in colorectal cancer cells. **a**, **c** HCT116 cells or SW480 cells were treated with 0.1 % DMSO or 5–50 μmol/L CPT for 24 h. p21 expression was analyzed by Western blot. Blots are representatives of three independent experiments. Time course activation of p21 in HCT1116 cells (**b**) or SW480 cells (**d**) treated with 25 μmol/L CPT from 0 to 36 h and analyzed by Western blot. Blots are representative of three independent experiments. **e** HCT116 cells or SW480 cells were treated with 0.1 % DMSO or 5–50 μmol/L CPT for 24 h. p27 expression was analyzed by Western blot. Blots are representatives of three independent experiments

addition, pharmacokinetic studies of CPT carried out in rats, mice, and pigs [40, 41] revealed that CPT is readily absorbed with an appreciable bioavailability with the highest plasma concentrations found in liver, lung, brain, and heart, with the lowest concentration in spleen and kidney [42]. We show that CPT can effectively hinder the cell viability of HCT116, SW480, and LOVO human colorectal cancer cells (IC50 12, 11, and 9 μM, respectively), with no apparent effect on the viability of non-tumor cells. CPT anticancer effects appear to be mediated by cell cycle arrest and induction of apoptosis in colorectal cancer cells with no evidence of apoptosis in non-tumor HEK293 cells. These results were accompanied by a significant reduction in colony formation on agar by CPT, indicating that CPT can prevent tumorigenesis in colorectal cancer cells. Here, we also report that CPT can inhibit the activation of Signal transducer and activator of transcription 3 (Stat3).

Cryptotanshinone inhibits Stat3 activation

Stat3 is a transcription factor that has become a suitable molecular target for cancer prevention and therapy. Stat3 activation is associated with poor prognosis in colorectal cancer [7]. Stat3 can regulate genes involved in proliferation, survival, tumor-associated inflammation, and angiogenesis, thus promoting tumorigenesis and cancer progression [43, 44]. Blockade of Stat3 was shown to induce apoptosis in colorectal cancer cells [45, 46] and retard tumor growth in colorectal cancer xenografts [46]. In our study, we found that CPT inhibits Stat3 activation in HCT116 and SW480 colorectal cancer cells in a dose- and time-dependent manner. This is consistent with that of previous reports that demonstrates CPT ability to rapidly inhibit Stat3 activation through JAK2-independent mechanisms in glioma cancer cells [19], lung cancer cells [47], and prostate cancer

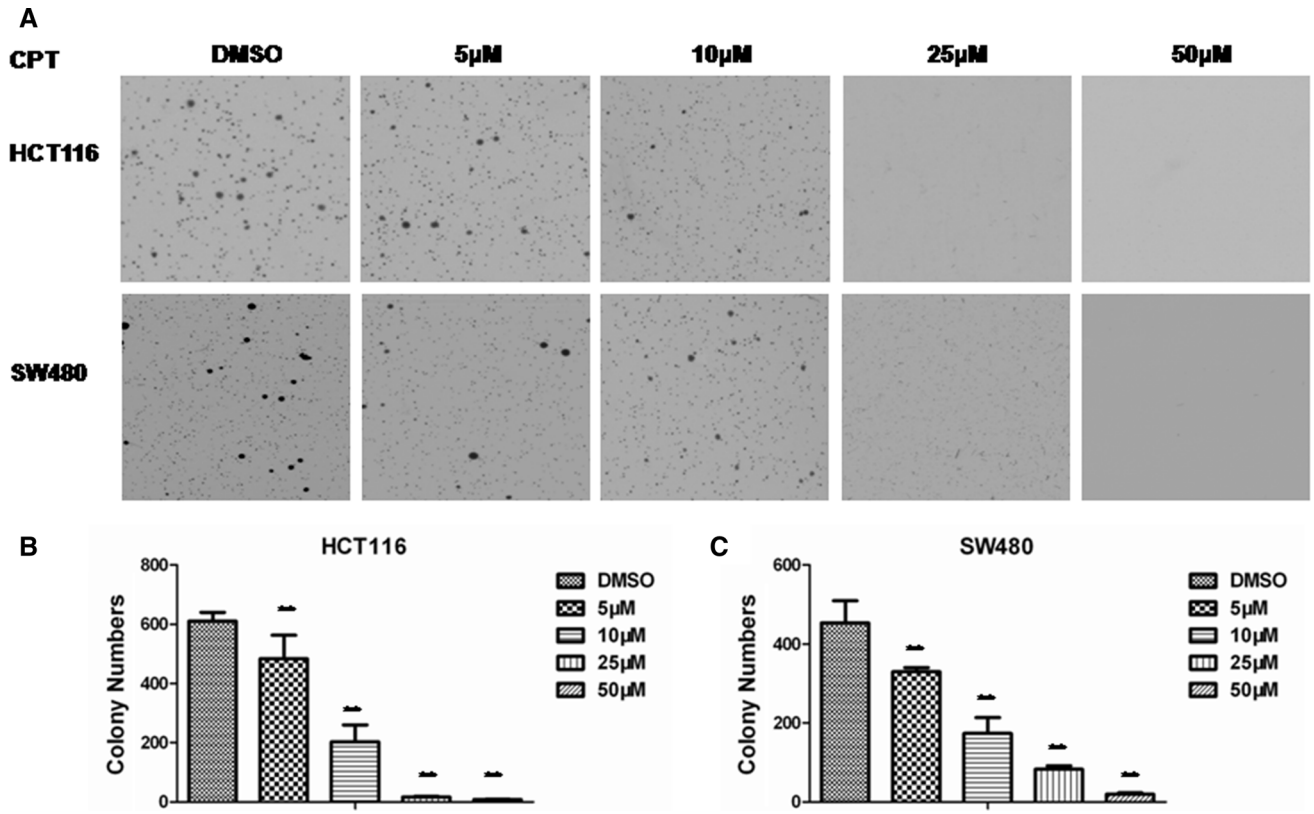

Fig. 6 Cryptotanshinone inhibits colorectal cancer cells anchorage independent growth. **a** HCT116 cells or SW480 cells were treated with vehicle (0.1 % DMSO) or 5–50 μmol/L CPT, for 10 days in soft agar, and the average colony numbers were counted. **b** and **c** show the colony numbers in HCT116 cells or SW480 cells treated by CPT, Column, mean of triplicate samples, bars, standard deviation, $^{**}p < 0.01$ versus DMSO control cells. Vehicle (0.1 % DMSO), *CPT* Crypotanshinone

cells [13]. In the case of prostate cancer cells, the mechanism involved the direct binding to the Stat3 monomer preventing downstream dimerization [13]. CPT appears to target the activation of endogenous Stat3, thus should predominately target cancer cells that have higher activation of Stat3 and minimal effect on non-tumor cells (HEK293) cells that have limited Stat3 activity unless in the presence of pro-inflammatory cytokines [6]. EGFR is an upstream regulator of Stat3 activation [48]. Consistent with the proposed mechanism of action of CPT by direct interaction with Stat3, we observed only a minor inhibition of EGFR phosphorylation at higher dosages of CPT (Fig. 4), thus suggesting that pSTAT inactivation by CPT is downstream of EGFR and independent of EGFR inactivation.

Cryptotanshinone blockade of Stat3-related targets and cell apoptosis

Stat3 signaling is involved in cancer progression by upregulating genes promoting cell cycle progression (cyclin D1 and c-myc) and/or preventing apoptosis (Bcl-xL, Bcl-2, and Survivin) [20, 49–51]. In our study, we found that CPT reduced the expression level of anti-apoptosis proteins, Bcl-2, CyclinD1, and Survivin in HCT116 and SW480 colorectal cancer cell lines. Apoptosis induction by CPT was confirmed using FITC Annexin V apoptosis detection assay and by activation of caspase-3. $p21^{WAF1/CIP1}$ and p27 are important inhibitors of cell proliferation by its interactions with complexes of cyclins and cyclin-dependent kinases (CDK) [52] and are also involved in the regulation of apoptosis [53]. Our results show that CPT can also induce p21 and p27 expression in HCT116 and SW480 colorectal cancer cells. The down-regulation of Bcl-2 and Survivin may be linked with the ability of CPT to induce cell death in colorectal cancer cells. Further studies using constitutively active Stat3 cell lines are necessary to confirm that CPT-mediated Stat3 inhibition is responsible for the precise apoptotic induction seen with CPT treatment.

Possible role of CPT in combination therapies

The effect of CPT on cell growth was evaluated in the present study by performing soft agar assays. This traditional method has been widely published and delivers

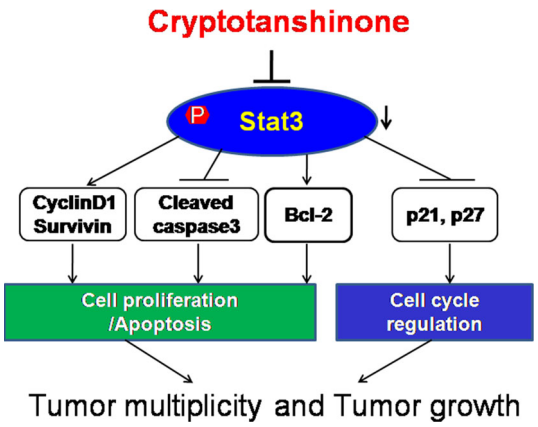

Fig. 7 Proposed mechanisms of action of CPT in suppressing colorectal cancer. CPT inhibits Stat3 phosphorylation and downstream targets CyclinD1, Survivin, and Bcl-2 and stimulates the cleavage of caspase-3. CPT treatment also induces p21 and p27, proteins involved in modulating cell cycle arrest. Interactions leading to activation of molecular targets are indicated by *arrows*; those that inhibited are indicated by a *bar*

results that are predictive of those seen in xenograft studies [54, 55]. Notably, in the present study, incubation with 5 μM of CPT alone provided significant inhibition of colony formation by both HCT116 and SW480 colorectal cell lines. There are currently a number of drug combinations approved for the treatment of colorectal cancer; however, despite the therapeutic potential, serious adverse effects can limit the use of most drugs. CPT may be a potential agent to sensitize tumor cells to be combined with conventional therapies. CPT has been shown to suppress doxorubicin efflux in doxorubicin-resistant HEPG2 hepatic liver cells [56] and radio sensitize HeLA cervical cancer cell lines [37, 57]. In the near future, we plan to investigate whether CPT can have synergistic effects in colorectal cancer cells with conventional chemotherapy drugs.

In summary, Stat3 has emerged as one of the most attractive targets for the treatment of cancer. Our findings demonstrate the anticancer activity of CPTin colorectal cancer cell line. CPT has been previously shown to directly bind to Stat3 inhibiting its activity in prostate cancer cells [13], and our study demonstrates that CPT can attenuate the phosphorylation of Stat3 in colorectal cancer cells. CPT also inhibited stat3-related gene products, including cyclinD1, Bcl-2, and Survivin in colorectal cancer cell lines (Fig. 7). The specificity of CPT in affecting the viability of tumor cells sparing non-tumor cells together with reported low patient toxicity makes CPT a strong candidate for combination therapy for the treatment of colorectal cancer.

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Conflict of interests The authors declare that there are no conflicts of interest.

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