Cryptotanshinone Inhibits Ovarian Tumor Growth and Metastasis by Degrading c-Myc and Attenuating the FAK Pathway

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Abstract

Background

Cryptotanshinone (CT), a natural compound derived from *Salvia miltiorrhiza* Bunge (Danshen, a traditional Chinese medicine), has been shown antitumor activity in various cancers. However, it remains unclear whether CT has a potential therapeutic benefit against ovarian cancers. The aim of this study was to test the efficacy of CT in ovarian cancer cells *in vitro* and using orthotopically implanted HEY A8 human ovarian cancer cells into NSG mice to determine its antitumor efficacy and explore the molecular mechanism underlying CT's antitumor effects.

Results

We found that CT inhibited the proliferation, migration, and invasion of OVCAR3 and HEY A8 cell while sensitizing the cell responses to chemotherapy drugs paclitaxel and cisplatin. CT also suppressed ovarian tumor growth and metastasis in immunocompromised mice orthotopically inoculated with HEY A8 cells. Mechanistically, CT degraded oncogene c-Myc by promoting ubiquitination and disrupting the interaction with its partner protein Max. CT also attenuated the FAK (focal adhesion kinase) signaling and degraded FAK in both cell lines. Knockdown of c-Myc using lentiviral CRISPR/Cas9 nickase resulted in reduction of FAK expression, which phenocopies the effects of CT and 10058-F4.

Conclusions

Our studies demonstrate that CT inhibits primary ovarian tumor growth and metastasis by degrading c-Myc and attenuating the FAK pathway.

Introduction

Ovarian cancer has the highest mortality rate among gynecological malignances with limited options of therapy (1). Currently the major strategy for treating ovarian cancer patients is to perform debulk surgery in combination with chemotherapy, targeted therapy, or hormone therapy. However, ovarian cancer frequently recurs due to chemotherapy drug resistance and metastasis(2-6). Therefore, it is essential to develop new medicine and improve efficacy and reduce cytotoxicity of chemotherapy drugs to overcome the chemoresistance. Natural products are an alternative resource for their antitumor activity and overcome multidrug resistance due to multiple potential gene targets and lower toxicity than conventional chemotherapy drugs (7, 8). At present, over 60% approved cancer drugs are derived from natural plants (9).

Cryptotanshinone (CT), a natural compound extracted from the root of *Salvia miltiorrhiza* Bunge (Red Sage), was previously reported to inhibit cell proliferation, motility/invasion, and angiogenesis by

targeting STAT3 in several cancers (10-16). However, whether CT has potential antitumor activity in ovarian cancer *in vivo* remains unknown, although some studies have reported its inhibitory effects on ovarian tumor cell growth *in vitro* (11, 17). The molecular mechanism underlying CT antitumor activity remains completely unknown.

The transcription factor c-Myc is upregulated or amplified in most cancer types including ovarian cancer (18). c-Myc is located in the same genomic locus (8q24) with another oncogene FAK (focal adhesion kinase), in a region known to be associated with aggressive cancer phenotypes, recurrence, and metastasis (19-21). Previous studies have shown that c-Myc and FAK was upregulated over 40% and amplified 65% in high grade serous carcinoma (HGSC), respectively, whereas p53 was mutated in over 90% patients (22). Therefore, targeting c-Myc and FAK represents a potential a novel strategy for ovarian cancer therapy. However, it is very difficult to directly target c-Myc using small molecule inhibitors due to its ubiquitous expression and cell nuclear localization. Several c-Myc inhibitors such as JQ1, 10058-F4, 10074-G5, and Omomyc have been developed for cancer therapy by targeting *Myc* transcription or interaction between c-Myc and its partner protein Max, thus blocking heterodimerization in the *Myc* promoter leading to inactivation of downstream target gene expression (23, 24).

The FAK pathway is activated in several different cancers, and multiple small molecule inhibitors to FAK have been developed. However, only four small molecule inhibitors, including GSK2256098, VS-4078, VS-6062, and VS-6063 (defactinib) have been used in clinical trials for cancer therapy (25-27). However, the most advanced compound VS-6063, failed in clinical trials to treat malignant pleural mesothelioma (28). FAK exerts its regulatory action either in a kinase activity-dependent or independent pathway, and these inhibitors only block FAK's kinase activity. However, these FAK kinase inhibitors do not degrade FAK, blocking its function as a scaffold protein. To fully block FAK's functions, a new generation drug PROTAC (Proteolysis Targeting Chimeric Molecule) was developed to inhibit both FAK's kinase activity and its scaffold protein function. PROTAC showed greater efficacy than its parent FAK inhibitor VS6063 in inhibiting tumor cell invasion (28).

Targeting both cMyc and FAK is important strategy for ovarian cancer therapy because both genes are highly amplified and upregulated. In this study, we assessed the antitumor effects of CT in ovarian cancer cells *in vitro* and tested its efficacy using orthotopic ovarian cancer mouse models *in vivo*. We uncovered a novel molecular mechanism underlying CT antitumor properties through degrading c-Myc and FAK.

Results

CT inhibits ovarian cancer cell proliferation and survival

To assess whether CT has cytotoxicity in ovarian cancer cells, we measured cell proliferation and viability in OVCAR3 and HEY A8 cells treated with different concentration of CT for 24 and 48 h. In both cell lines, CT inhibited the proliferation in a dose- and time- dependent manner (Fig. 1A & B). CT significantly inhibited cell proliferation and viability from 10 to 80 μ M after 24 h or 48 h treatment. We also examined

cell viability using acridine orange/propidium staining at the low doses of 10 and 20 μ M and found that CT did not induce significant cytotoxicity (Fig. S1A & S1B). We further assessed how CT affects colongenic survival using colony formation assay, both cell lines were treated with 0.5 μ M CT for seven days and CT significantly inhibited colony growth (Fig. 1C & 1D).

Ct Suppresses Ovarian Cancer Cell Migration And Invasion

Migration and invasion contribute to ovarian tumor metastasis. To examine whether CT regulates ovarian cancer cell migration and invasion, we treated OVCAR3 and HEY A8 cells with 20 μ M CT for 24 h. Cell migration was determined in transwell plates, whereas invasion was examined using Matrigel coated transwell inserts. CT significantly inhibited migration and invasion in both OVCAR3 and HEY A8 cells (Fig. 2A & 2B).

CT inhibits c-Myc and FAK expression comparable to that of c-Myc inhibitor 10058-F4

We examined c-Myc and FAK genetic alterations in patients with HGSC deposited in The Cancer Genome Atlas (TCGA) database. c-Myc and FAK were amplified or upregulated in 35% and 40%, respectively whereas p53 was mutated in 65% of 585 patients in TCGA Pan Cancer Atlas. c-Myc and FAK were amplified or upregulated in 44% and 43%, respectively while p53 was mutated in 49% of 594 patients in TCGA Firehouse Legacy (Fig. 3A). Therefore, c-Myc and FAK are potentially important drug targets for ovarian cancer therapy. Several c-Myc or FAK inhibitors are still in clinical trials. In this study, we found that CT has a potency in inhibiting both c-Myc and FAK expression, which was comparable to the c-Myc inhibitor 10058-F4 (29). To examine how CT regulates c-Myc and FAK, we treated OVCAR3 and HEY A8 cells with different doses of CT or 10058-F4 for 24 h and monitored c-Myc and FAK expression with Western blotting. The CT mediated c-Myc and FAK inhibition in a dose-dependent manner with significant inhibition manifesting at the low 10 -to-20 μ M doses (Fig. 3B). CT or 10058-F4 applied at 10 μ M inhibited both c-Myc and FAK expression in a time-dependent manner with a significant reduction detectable at 24 or 48 h in OVCAR3 and HEY A8 cells (Fig. 3C).

CT or knockdown of c-Myc attenuates RGD-induced FAK activation in ovarian cancer cells

Because CT mediated inhibition of c-Myc and FAK occurred after 12 h treatment, we assessed whether CT was able to block FAK activation at earlier time points. OVCAR3 and HEY A8 cells were treated with 10 μ M CT or 20 μ M FAK inhibitor GSK2256098 (30, 31) for 8 h. Subsequently the FAK pathway was activated using 20 μ M RGD (32), a small peptide ligand of integrin receptor at different time points. CT attenuated RGD-induced FAK activation but failed to induce complete FAK protein degradation (Fig. 4A & B). These effects were comparable to that of the FAK inhibitor GSK2256098 in both cell lines (Fig. 4C & D). We further tested whether a genetic approach with KD of c-Myc would phenocopy the pharmacological attenuation of FAK signaling. We established stable OVCAR3 and HEY A8 cell lines with c-Myc KD using lentiviral CRISPR/Cas9 nickase vector and treated these cells with 20 μ M RGD for different times. RGD activated FAK phosphorylation in control OVCAR3 and HEY 8 cells, but activation was significantly attenuated in c-Myc KD cells with reduced c-Myc and FAK expression (Fig. 4E & F). Overall, the

attenuation of FAK signaling and FAK degradation in c-Myc KD cells was consistent with the effects of CT in both cell lines; although, c-Myc and FAK degradation occurred at a later time following CT treatment.

CT is similarly effective as the c-Myc inhibitor 10058-F4 in sensitizing ovarian cancer cell responses to chemotherapy-induced apoptosis

Because CT targets c-Myc and FAK in ovarian cancer cells, we hypothesized that this drug might induce apoptosis by functioning like a c-Myc inhibitor (33). We examined the antitumor activity of CT in ovarian cancer cells and compared it with c-Myc inhibitor 10058-F4 by analyzing apoptosis by determining the levels of cleaved PARP and caspase3 using Western blotting. CT was not cytotoxic at 10 or 20 μ M concentration in ovarian cancer cells (Fig. S1A & 1B). Therefore, we treated OVCAR3 and HEY A8 cells with CT or 10058-F4 using different concentrations ranging from 5 to 20 μ M for 24 h. CT induced significant cleavage of PARP that was comparable to the cleavage elicited by 10058-F4 over the same concentration range in both cell lines. We detected only low levels of cleaved-PARP at the 5 μ M dose in HEY A8 cells (Fig. 5A). We also treated OVCAR3 and HEY A8 cells with 10 μ M CT or 10058-F4 for different times. Both CT and 10058-F4 induced significant increase in PARP cleavage at 24 or 48 h time points in OVCAR3 cells but only this response was delayed at 48h in HEY A8 cells. This indicated that HEY A8 cells are more resistant to CT and 10058-F4-induced apoptosis than OVCAR3 cells are (Fig. 5B).

To explore the potential therapeutic applicability of CT, we examined whether it displays a synergistic effect in inducing cell apoptosis when CT was administrated in combination with other chemotherapeutic drugs. OVCAR3 and HEY A8 cells were treated with CT and two commonly used chemotherapy drugs paclitaxel and cisplatin. Compared to vehicle, 10 μ M CT enhanced the efficacy of both paclitaxel (5 nM) and cisplatin (10 μ M) as shown by the increased levels of cleaved PARP and caspase-3 (Fig. 5C & 5D). We also examined apoptosis by performing immunofluorescent staining using the antibody that recognizes cleaved caspase3. The apoptotic cell nuclei were present in significantly higher numbers in cells treated with a combination of paclitaxel or cisplatin with CT compared with cells treated with CT, paclitaxel, or cisplatin alone (Fig. S2A & 2B). Our data indicated that CT enhanced ovarian cancer cell death induced by paclitaxel and cisplatin treatment.

CT degraded c-Myc through increased ubiquitination by disrupting the interaction between Max and cMyc in ovarian cancer cells

To understand the molecular mechanisms underlying CT mediated c-Myc degradation, we treated OVCAR3 cells with proteasome inhibitor MG132. MG132 applied at a 5 μ M blocked c-Myc degradation induced by CT to an extent that was comparable to that of 10058-F4 (Fig. 6A). We further assessed c-Myc stability using a cycloheximide chase assay and found that the half-life of c-Myc was significantly reduced from 41 to 36 min by CT treatment in OVCAR3 cells, whereas the control drug 10058-F4 reduced it from 43 min to 34 min. These results indicate that CT promoted c-Myc proteasomal degradation similar to the c-Myc inhibitor (Fig. 6B). We examined whether c-Myc degradation occurs through the ubiquitination pathway in ovarian cancer cells. We treated OVCAR3 cells with 10 μ M CT for 24 h and

detected ubiquitination using ubiquitin antibody. We detected increased ubiquitination in CT-treated compared to vehicle treated control cells by Western blot using ubiquitin antibody. CT induced ubiquitination was also validated by fluorescent staining, and we observed stronger staining in CT-treated than vehicle treated cells (Fig. 6C). We further examined whether CT mediated ubiquitination through disrupting the interaction between c-Myc and Max, which is required for cMyc stability. Thus, we treated OVCAR3 cells with 10 μ M CT for 24 h and co-immunoprecipitated with its partner Max antibody. The immunocomplex was blotted with cMyc antibody. As we expected, we were unable to detect cMyc expression in CT treated cells but detected cMyc expression in vehicle treated cells, indicating that CT disrupted the interaction between cMyc and its partner Max. To detect CT induced cMyc polyubiquitination, we treated OVCAR3 for 6h with 10 μ M CT and 5 μ M MG132, protein complex was immunoprecipitated with cMyc antibody and detected then by ubiquitin and cMyc antibody using WB. The ubiquitinated cMyc was clearly detected by ubiquitin antibody in OVCAR3 cells treated with CT, MG132 or MG132 and CT (Fig.S3). These data indicate that CT degraded c-Myc by disrupting cMyc stability through the ubiquitination pathway.

CT inhibits primary ovarian tumor growth and metastasis in vivo

To test the efficacy of CT in ovarian tumor therapy, we injected WT HEY A8 cells intrabursally into NSG female mice and treated them intraperitoneally with CT (10 mg/kg bodyweight) or vehicle for three weeks. Primary tumors in ovaries were significantly reduced in CT-treated mice compared to vehicle-treated mice as shown by bioluminescence in live mice (Fig. 7A) and in dissected ovaries (Fig. 7B). Ovarian tumors were observed in vehicle-treated mice but were reduced in size in HE-stained ovaries of CT-treated mice (Fig. 7C). In addition, we examined the level of c-Myc and FAK in primary ovarian tumors using Western blot analysis. c-Myc and FAK were both significantly reduced in ovarian tumors in mice treated with CT compared with control mice (Fig. 7D). We also examined tumor metastasis in this animal model and found CT significantly inhibited tumor metastasis in multiple peritoneal organs including the liver compared to vehicle-treated mice (Fig. 7E & F). These results demonstrated that CT suppressed primary ovarian tumor growth and metastasis by inhibiting c-Myc and FAK.

Discussion

CT as a natural compound with demonstrated antitumor activity in a variety of cancers. In this study, we demonstrated for the first time that CT inhibits HEY A8 ovarian tumor growth and metastasis in an orthotopic mouse model. In particular, we defined a novel molecular mechanism underlying CT's antitumor activity by demonstrating degradation of c-Myc through ubiquitination. We found that CT attenuated FAK signaling at an early time point and degraded FAK at a later time period. Although we choose two different cell lines with different p53 status, we found no impact of p53 expression on CT treatment (data not shown).

CT induced apoptosis in HEY A8 and OVCAR3 cell lines. However, we found that the HEY A8 cell line is more resistant to CT- and 10058-F4-induced apoptosis compared to OVCAR3 cells. We also found that

HEY A8 cells are more aggressively metastatic than OVCAR3 cells in our orthotopic mouse models. It is for this reason that we chose the HEY A8 line to test the therapeutic efficacy of CT. Although p53 is wild type in HEY A8, whereas mutated in OVCAR3 cells, our data supported that the efficacy of CT was independent on the p53 status of these cell lines. We also showed that CT displayed a synergistic effect in promoting paclitaxel and cisplatin-induced cell apoptosis, suggesting that CT is a bona fide antitumor agent when applied in combination with this chemotherapeutics. CT inhibited ovarian cancer cell proliferation but only above concentrations above 10 μ M. Our experiments demonstrated that CT controls tumor cell growth by inhibiting proliferation, migration, invasion, and also induces apoptosis. In our orthotopic ovarian cancer mouse models, CT inhibited primary ovarian tumor growth and metastasis. From a therapeutic perspective, we note that CT enhances the efficacy of chemotherapeutic drugs used in the treatment of ovarian cancer (Fig. 4). However, the potency of CT in overcoming chemoresistance using ovarian cancer chemotherapy drug resistant cell lines *in vitro* and also in mouse models *in vivo* remains to be tested.

c-Myc and FAK were highly expressed in both our cell lines. Based on the aberrant expression of c-Myc and FAK in ovarian cancer, it is important to target both genes. Interestingly, we observed that CT degraded both c-Myc and FAK in ovarian cancer cells after 12 h treatment, which is comparable to the effects of the Myc inhibitor drug 10058-F4 (Fig. 3). We detected the mRNA expression of c-Myc and FAK using real-time RT-PCR (data not shown) but did not find any significant differences between CT treated compared to vehicle treated cells, suggesting that c-Myc and FAK degradation may be regulated at the posttranscriptional level. MG132 inhibited CT-induced c-Myc degradation, indicating that it may degrade c-Myc via the ubiquitination pathway. Indeed, our results showed that CT promoted ubiquitination in ovarian cancer cells. However, CT did not affect the level of expression of c-Myc binding partner Max. Co-immunoprecipitation using a Max antibody showed that CT may disrupt the heterodimerization with Max in vehicle-treated cells but not in the CT-treated cells. This indicates that CT potentially targets c-Myc by disrupting its stability.

Although we observed that CT degraded c-Myc and FAK in both cell lines regardless of their p53 status, it remains unclear whether CT directly degrades FAK or degrades c-Myc which subsequently leads to a decrease in FAK. To answer this question, we knocked down c-Myc using lentiviral CRISPR/Cas9 nickase and found that KD of c-Myc resulted in reduction in FAK protein. This suggests that FAK expression appears to be regulated by c-Myc, and FAK reduction might be a result of c-Myc degradation. Previous studies showed that N-Myc transcriptionally regulated FAK expression by binding to the FAK promoter in neuroblastoma cells (34). Therefore, it is plausible that c-Myc transcriptionally activates FAK expression, leading to FAK upregulation in ovarian cancer cells. We failed to detect a direct interaction between c-Myc and FAK through co-IP, though FAK is known to be functional as a scaffold protein in addition to its kinase activity.

Conclusions

We demonstrated that CT is a potential antitumor drug candidate in ovarian cancer therapy not only because it improves the efficacy of other chemotherapeutic drugs but more importantly because it causes the degradation of c-Myc by disrupting its stability and attenuating the FAK pathway.

Materials And Methods

Cell culture

Two different ovarian cancer cell lines, OVCAR3 and HEY A8 were chosen for this study due to their different p53 status. p53 was mutated in OVCAR3 cells and was wildtype (WT) in HEY A8 cells. Both cell lines were purchased from National Cancer Institute (Bethesda, MD) and cultured in IM1640 medium supplemented with 10% fetal bovine serum (FBS, Hyclone; Logan, UT), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen; Carlsbad, CA).

MTT cell proliferation assay

The MTT cell proliferation assay kit was purchased from ATCC and used to measure cell proliferation and viability. OVCAR3 or HEY A8 cells were seeded to 96-well plates at 3,000 cells/well density and incubated overnight. The next day, cells were treated with 0.1% DMSO or different doses of CT and cultured for 24 or 48 h. MTT reagent (10 µl) was added to each well and incubated for 4 h. The reaction was stopped by adding 150 µl DMSO and the plates were incubated at room temperature in the dark for 10 min. Cell proliferation was assessed by measuring the absorbance at 570 nm. Cell viability was also measured with acridine orange/propidium solutions. Live green and dead red cells were counted using a Luna-FL automatic cell counter.

Colony formation assay

Three hundred ovarian cancer cells per well were plated and cultured for 72 h before treatment. Subsequently, the cells were treated with DMSO or 0.5 µM CT in triplicate. Seven days later, the colonies were fixed and stained with crystal violet (Sigma, St. Louis, MO, USA) dissolved in 10% ethanol. The number of colonies was counted.

Cell migration and invasion assay

Briefly, OVCAR3 or HEY A8 cells were treated with 20 µM CT or 0.1% DMSO for 24 h. The modified Transwell chambers (BD Falcon™, San Jose, CA) were inserted into 24-well cell culture plates. IM1640 medium containing 10% FBS served as the chemoattractant in the lower chamber. Then 3×10^5 cells in 300 µl serum-free IM1640 were added to the upper chamber coated with or without Matrigel as described previously for migration and invasion assays, respectively [8]. After incubation for 24 h, the non-migrated and invaded cells were removed gently with a cotton swab. The migrated or invaded cells located on the lower surface of chamber were stained with crystal violet or hematoxylin-eosin (HE) and counted.

Immunofluorescence assay

Following exposure to 0.1% DMSO, 10 μ M CT, or CT combined with chemotherapy drugs for 24 h, the cells were washed with PBS, fixed in 99.8% methanol for 20 min and permeabilized with 0.1% Triton X-100 in PBS containing 0.5% BSA (Sigma-Aldrich) for 1 h. To detect the expression of cleaved caspase-3 in cells, the cells were fixed in 4% (W/V) paraformaldehyde (PFA) and incubated with blocking buffer containing 5% normal goat serum in PBS for 1 h. The cells were subsequently incubated with cleaved caspase-3, c-Myc, and ubiquitin antibodies (Cell Signaling, diluted 1:200 in blocking buffer) overnight at 4 °C. After washing with PBS, cells were incubated with Alexa Fluor 488- or Alexa Fluor 568-conjugated secondary antibody (A27023; 1:1,000 Thermo Fisher Scientific, Inc.) at room temperature in the dark for 45 min. The cells were washed with PBS, incubated in the 100ng/ml DAPI solution, and imaged under a fluorescent microscope.

Generation of c-Myc knockdown (KD) ovarian cancer cells using lentiviral CRISPR/Cas9 nickase vector

The lentiviral CRISPR/Cas9 nickase-mediated c-Myc gene editing vectors were constructed by synthesizing and annealing two gRNAsc (5'TTGGTGAAGCTAACGTTGAG and 5'CTATGACCTCGACTACGACT) and subcloning them in the BsmII site of lentiviral Lentiguide-puro vector (#52963, Addgene, Watertown, MA). CRISPR/Cas9 nickase was driven by EF1a promoter in LentiCas9-blast vector (catalog #52962, Addgene). Lentivirus was produced by packaging in 293FT cells as described previously (35). Stable c-Myc KD cell lines were established by transducing the OVCAR3 or HEY A8 cells LentiCas9-blast Cas9 nickase vector and selected in 10 μ g/ml blasticidin, and subsequently transduced with the lentiviral CRISPR/Cas9 nickase-mediated c-Myc gRNA vector and selected with 1 μ g/ml puromycin. LentiCas9-blast was used as the control vector without gRNAs.

Western blot

Western blot analysis was performed as previously described (36). In the MG132 proteasome inhibitor experiments, cells were treated with 10 μ M CT and 5 μ M MG132 (Medexpress, Monmouth Junction, NJ) for 24 h and collected for Western blot analysis. In the cycloheximide (CHX, Medexpress) chase assay, cells were treated with 10 μ M CT for 24 h, when 50 μ g/ml of CHX was added and cells were collected at different time points. Primary antibodies included cleaved-PARP, cleaved-caspases-3, c-Myc, Max, FAK, p-FAK (1:1000, Cell Signaling), Ubiquitin, and GAPDH (1:1000, Santa Cruz).

Co-Immunoprecipitation Assay (Co-IP)

To detect the physical interaction between cMyc and Max, OVCAR3 cells were treated with 10 μ M CT for 24 h and to detect the ubiquitinated *cMyc*, and prevent CT induced *cMyc* degradation, OVCAR3 cells were treated with 10 μ M CT and 5 μ M MG132 for only 6h and lysed with 25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol buffer, pH 7.4). The cell lysates were pre-cleared with Control Agarose Resin from Pierce Classic IP Kit (Thermo, 26146) for 1 h at 4 °C. 1.5 mg of protein from the lysate was incubated with either or Max antibody (Cell Signaling) or c-Myc antibody (Santa Cruz, N-262) overnight at 4 °C. Then the immune complex was captured with Pierce Protein A/G Agarose for 2 h and washed three times with lysis buffer, one time with conditioning buffer, and eluted with elute buffer included in the kit.

The eluted fractions were then analyzed by Western blot using cMyc or ubiquitin (Santa Cruz, SC-8017) antibodies, respectively

Orthotopic ovarian cancer mouse model

All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center. Two-month-old immunocompromised NOD Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) female mice were purchased from the Jackson Laboratory. HEY A8 cells were labeled with luciferase by using the lentiviral vector pEF1a-Luc2. 5×10^5 cells were injected intrabursally into the NSG females under dissecting microscopy. After one week, mice were randomized into groups of five and treated with vehicle (10% DMSO + 90% corn oil) and or treated with CT (10 mg/kg bodyweight) via intraperitoneal injection for five days a week for three weeks. Primary ovarian tumor growth and metastatic dissemination in NSG mice were monitored weekly using live animal PerkinElmer Imaging system by measuring bioluminescence following intraperitoneally injection of 0.25 ml of 15 mg/ml D-luciferin. All mice were sacrificed after three weeks. Tumors were harvested for histology, immunofluorescent staining, and Western blot to determine c-Myc and FAK expressions.

Statistical analysis

Significant differences were determined from at least three independent experiments performed in triplicate by Student's *t*-test, and data were presented as mean $\pm$ SD. $p < 0.05$ was considered significant.

List Of Abbreviations

CT
Cryptotanshinone
HGSC
high grade serous carcinoma
FAK
focal adhesion kinase
PROTAC
Proteolysis Targeting Chimeric Molecule
TCGA
The Cancer Genome Atlas
WT
wildtype
FBS
fetal bovine serum
HE
hematoxylin-eosin
PFA

paraformaldehyde
KD
knockdown
CHX
cycloheximide
IACUC
Institutional Animal Care and Use Committee
NSG
NOD Cg-Prkdcscid Il2rgtm1Wjl/SzJ.

Declarations

Ethics approval and consent to participate: All animal experiments were conducted in accordance with the approval from the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests: Authors have no conflict of interest to declare.

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Author's contributions: **Yue J, Zhang W, Zhu BM, Dong P:** Conceptualization, Methodology. **Guo H, Zhao G, Wang Y:** Data curation, Investigation. **Yue J, Zhang W, Zhu B:** Supervision. **Zhao G,** Validation. **Yue J, Guo H, Dong P, Watari H, Tigyi G:** Funding acquisition, Writing- Reviewing and Editing.

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Figures

Figure 1

CT inhibits ovarian cancer cell proliferation. (A), (B) MTT proliferation assay in OVCAR3 (A) and HEY A8 cells (B) treated with different doses of CT for 24 and 48 h. (C), (D) Colony formation assay of OVCAR3 and HEY A8 cells treated with 0.5 μM CT treatment for seven days in 6-well plates. Representative images and average colony numbers were shown. Data were presented as mean ± SD. *p< 0.05, **p < 0.01, versus control group (n = 3).

Figure 2

CT inhibits ovarian cell migration and invasion. (A) Transwell migration assay was performed in OVCAR3 and HEY A8 cells treated with 20 μ M CT treatment for 24 h. Migrated cells were stained using crystal violet and counted. (B) Matrigel-coated transwell assay was performed in OVCAR3 and HEY A8 cells treated with 20 μ M CT for 24 h. Invaded cells were stained using H.E. and counted. Representative images were shown. Quantification of migration and invasion was presented as mean $\pm$ SD from three independent experiments. (* p <0.05, ** p <0.01).

Figure 3

CT inhibits c-Myc and FAK expression in ovarian cancer cells. (A) c-Myc and FAK were amplified or upregulated in HGSC in two different datasets. The alteration for each gene was indicated as percentage among those patients. (B) Western blot and densitometry analysis of c-Myc and FAK expression following different doses of CT or 10058-F4 treatment for 24 h in OVCAR3 and HEY A8 cells. (C) Western blot and densitometry analysis of c-Myc and FAK expression in OVCAR3 and HEY A8 cells following 10 μ M CT or 10058-F4 treatment at different time points. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 4

CT or KD of c-Myc attenuates RGD-induced FAK activation in ovarian cancer cells

(A, B) Western blot and densitometry analysis of p-FAK and total FAK following 10 μ M CT treatment for 8h and then 20 μ M RGD treatment at different time points in OVCAR3 (A) and HEY A8 cells (B). (C, D) Western blot and densitometry analysis of p-FAK and total FAK following 20 μ M GSK2256098 treatment for 8 h and then 20 μ M RGD treatment at different time points in OVCAR3 (C) and HEY A8 cells (D). (E, F) Western blot and densitometry analysis of p-FAK, total FAK and c-Myc expression in c-Myc KD and control OVCAR3 (E) and HEY A8 (F) cells following 20 μ M RGD treatment at different time points (* p <0.05, ** p <0.01, *** p <0.001).

Figure 5

CT sensitizes chemotherapy drug induced apoptosis in ovarian cancer cells. (A, B) Western blot and densitometry analysis of apoptosis in OVCAR3 and HEY A8 cells by examining cleaved-PARP following different doses (A) of CT or 10058-F4 treatment for 24 h or different time points with the same dose of 10 μM (B). (C, D) Western blot and densitometry analysis of apoptosis in OVCAR3 (C) and HEY A8 cells (D) by detecting cleaved-PARP and cleaved-caspase3 following CT treatment with paclitaxel and cisplatin together or alone for 24 h. One representative Western blot was presented from three similar independent experiments. The expression of cleaved-PARP and cleaved-caspase3 were compared between CT or 10058-F4 and vehicle treated cells (*p<0.05, **p<0.01, ***p<0.001).

Figure 6

CT degrades c-Myc through ubiquitination pathway. (A) c-Myc expression was determined by Western blot in OVCAR3 cells treated with 10 μM/L CT or 10058-F4 with or without the proteasome inhibitor MG132. (B) OVCAR3 cells were pretreated with 10 μM CT or DMSO for 24 h, followed by 10 μg/ml CHX treatment and c-Myc expression was detected by Western blot. (C) Western blot analysis of ubiquitination in vehicle and CT treated OVCAR3 cells and immunofluorescent staining of c-Myc (Red) and ubiquitin (green) in OVCAR3 cells following 24 h CT or vehicle treatment. (D) Western blot analysis of c-Myc and Max, following co-immunoprecipitation using MAX antibody in OVCAR3 cells treated with CT or vehicle for 24 h. (Scale bar, 5 μM. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 7

CT inhibits ovarian tumor growth and metastasis. (A, B) Bioluminescence of live imaging of mice (A) and primary ovarian tumors (B) at three weeks following CT or vehicle treatment in NSG mice intrabursally injected with WT HEY A8 cells (n=5, *p<0.05, **p<0.01). (C) Sections of primary ovarian tumors were stained with immunofluorescence and H. E. (D) Western blot and densitometry analysis of c-Myc and FAK expression in primary tumor from three different xenografted mice treated with CT and vehicle (*p<0.05, **p<0.01). (E) Metastatic tumors in liver of mice treated with CT treatment and vehicle. (F) Sections of metastatic tumors in liver were stained with H. E. (*p<0.05, **p<0.01).

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