RESEARCH ARTICLE

Cryptotanshinone Induces Inhibition of Breast Tumor Growth by Cytotoxic CD4+ T Cells through the JAK2/STAT4/ Perforin Pathway

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Abstract

Cryptotanshinone (CPT), is a quinoid diterpene isolated from the root of the Asian medicinal plant, *Salvia miotiorrhiza* bunge. Numerous researchers have found that it could work as a potent antitumor agent to inhibit tumor growth *in vitro*, buith there has been much less emphasis on its *in vivo* role against breast tumors. Using a mouse tumor model of MCF7 cells, we showed that CPT strongly inhibited MCF7 cell growth in vivo with polarization of immune reactions toward Th1-type responses, stimulation of naive CD4+ T cell proliferation, and also increased IFN-γ and perforin production of CD4+ T cells in response to tumor-activated splenocytes. Furthermore, data revealed that the cytotoxic activity of CD4+ T cells induced by CPT was markedly abrogated by concanamycin A(CMA), a perforin inhibitor, but not IFN-γ Ab. On the other hand, after depletion of CD4+ T cells or blocked perforin with CMA in a tumor-bearing model, CPT could not effectively suppress tumor growth, but this phenomenon could be reversed by injecting naive CD4+ T cells. Thus, our results suggested that CPT mainly inhibited breast tumor growth through inducing cytotoxic CD4+ T cells to secrete perforin. We further found that CPT enhanced perforin production of CD4+ T cells by up-regulating JAK2 and STAT4 phosphorylation. These findings suggest a novel potential therapeutic role for CPT in tumor therapy, and demonstrate that CPT performs its antitumor functions through cytotoxic CD4+ T cells.

Keywords: CPT - perforin - cytotoxic CD4+ T cells - breast tumor - JAK2 - STAT4

Asian Pac J Cancer Prev, **15 (6)**, 2439-2445

Introduction

Cryptotanshinone (CPT) is one of the major tanshinones isolated from Salvia miltiorrhiza Bunge (Danshen), which has been showed to inhibit the growth of multiple kinds of tumor cells, or induced tumor cells apoptosis in *in vitro* experiments, including prostate cancer (Shin et al., 2009), leukemia (Kim et al., 2011), lung cancer (Lee et al., 2008), breast cancer (Park et al., 2012), and cervical cancer (Ye et al., 2010). Until now, researchers have found that CPT down-regulate cyclin D1 expression, which results in cell-cycle arrest in G1 phase (Chen et al., 2010), it also inhibits human glioma cell proliferation by suppressing STAT3 signaling (Lu et al., 2013); with regard to apoptosis, CPT sensitizes Fas or ER stress-mediated or enhanced TNF-α-induced apoptosis (Park et al., 2010; Kim et al., 2011; Park et al., 2012).

However, these results all got from *in vitro*, and antitumor effects of CPT still didn't be identified in animal model. Considered that breast cancer is the most prevailing disease among all cancers (Soerjomataram et al., 2012), and previous reports have showed that CPT induces ROS release which activates p38/JNK and inhibits Erk1/2, leading to caspase-independent cell death of human tumor cells (Rh30, DU145, and MCF-7) (Chen et al., 2012), we wondered is that possible CPT inhibits MCF7 cells grow *in vivo*? If so, how does it perform its antitumor function?

To test this possibility, we constructed a mouse tumor-bearing model, and found that CPT significantly inhibited the growth of MCF7 cells *in vivo*. To further identify how CPT accomplish this function, we analyzed multiple indicators with tumor-eradiated activity, our results showed that, besides polarized immune reaction towards th1 subtype, CPT obviously enhanced perforin production of CD4+ T cells, but played a minor role on CD8+ T cells. If we firstly blocked perforin secretion by Concanamycin A (CMA) *in vivo*, the antitumor activity of CPT was diminished, unless followed with adoptive

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transferring congeneric CD4+ T cells, this phenomenon could be reversed. So we concluded that CPT mainly depended on Cytotoxic CD4+ T cells to achieve its antitumor function.

According to Yamamoto K's group report (Yamamoto et al., 2002), perforin gene is a direct target of STAT4 activated by IL-12, beside this, Lesley White's groups also found that IL-21 up-regulated perforin expression in CD8+ T cells (White et al., 2007), as a fact that CPT stimulated perforin production of CD4+ T cells, CPT may work like IL12/IL-21 to stimulate perforin production of CD4+ T cells through STAT4. In view of JAK2/STAT4 pathway has been found to be related with the proliferation and activation of T cells (Bright et al., 1998; Collins et al., 1999), CPT may positively regulate JAK/STAT pathway to activate CD4+ T cells secrete perforin. To test this hypothesis, we detect JAK2 and STAT4 expression level and their phosphorylation in CPT activated CD4+ T cells, results showed that CPT work as a positive regulator of JAK2/STAT4 pathway to stimulate CD4+ T cells secrete perforin and therefore inhibit tumor growth.

Materials and Methods

Materials

Reagents like CPT, CMA, Taxol and AG490 all purchased from Sigma-Aldrich (St Louis, MO), Antibodies including PE-anti-mouse IFN-γ/perofin/ IL-10/IL-4/granzyme B, APC-anti-mouse CD3, FITC-anti- -mouse CD8 /CD4, as well as IFN-γ and CD4 Ab used for neutralization assay were purchased from BioLegend. Mouse IFN-γ/IL-10/IL-4 ELISA kits were from DAKEWE (Shenzhen, China), pSTAT4Tyr693 Alexa Fluor 488 and CD4/CD8 T cell magnetic isolation kits were purchased from BD Bioscience. JAK/pJAK Abs and LDH- -Cytotoxicity Assay Kit II were from Abcam (Abcam Inc., Cambridge, MA, USA). Wild-type C57BL/J6 mice (4-6 weeks old, female) were from Hubei Province Centre for Disease Control and Prevention. The animal experimental protocols were performed in compliance with all guidelines and were approved by the Institutional Animal Care and Use Committee of Wuhan University.

Tumor challenge and treatment. MCF7 cells in the exponential growth phase were harvested and washed in PBS, before *in vivo* injection, their viability exceeded 95%, as determined by trypan blue staining. To induce localized tumors, syngeneic C57BL/J6 mice were sub-cutaneously (s.c.) challenged into the forth Mammary Gland with 1×10^6 MCF7 breast cancer cells (Murphy et al., 1993). CPT in 0.1% solution of sodium lauryl sulfate was administered orally at 150 mg/kg body weight/day in the morning (9:00 to 10:00 AM) (Kim et al., 2007) since day 5, Taxol (3 μg/g) and CMA (12 ng/g) (Hettiarachchi et al., 2006) were administered i.*p*. once a week. Tumor size was determined every four day in 30-day interval with caliper, and the tumor volume in mm3 was calculated by the formula: Volume= $(\text{width})^2 \times \text{length}/2$.

Cell proliferation

Cells proliferation was detected by CCK-8 assay. Freshly isolated CD4+ T cells from splenocytes of WT mice, and then seeded 1×10^5 cells in 100 μl (CD4+ T cells/ splenocytes /MCF7 cells) into 96-well plate. CPT was added to cell media at different concentrations from 1.25 to 40 μM, simultaneously added 10 μl CCK-8 solution. Detected OD450 after 4 hours

CD4+ lymphocyte depletion

Tumor-bearing mice were depleted of CD4+ T cells by intraperitoneal (i.*p*.) injections of 100 μg of the anti-CD4 antibody 2 days before tumor cells inoculation. The efficacy of the depleting treatment was confirmed by flowcytometry that Depleted populations contained <0.25% CD4+ cells in splenocytes (Schoenberger et al., 1998).

Serum cytokines measurement.

For *in vivo* analysis of level of IFN-γ/IL-4/ IL-10/ perforin in serum, the serum of each mouse group was harvested at day 30 post injected tumor cells, and then collected for analyzing cytokine levels by using an Ag-capture ELISA specific kits according to the protocol recommended by the manufacturer.

Flow cytometry.

For T-cell cytokines staining, splenocytes, or other cell preparations were incubated with anti-mouse CD16/CD32 (eBioscience) in the room temperature for 10 min, and then directly conjugated surface APC-CD3, FITC-CD4/CD8 for 30 min at 4 °C. After that cells were treated with fixation solution (biolegend) for 20 min at room temperature. Cells were washed twice with permeabilization buffer and then stained with anti-mouse IFN-γ or other cytokine antibodies in permeabilization buffer (30 min at 4 °C) before analysis. All directly conjugated antibodies were from Biolegend unless mentioned otherwise. Before harvested, cells were incubated for 5h in the presence of PMA (50ng/ml; Sigma), ionomycin (500ng/ml; Sigma) and Brefeldin A Solution (1000×; Biolegend) (Wang et al., 2013). The intracellular cytokines production was detected on a BD C6 flow cytometer.

In vitro Cytotoxicity assay

Target cells (MCF7 cells) were prepared at 1×10^4 cells/ ml, CD4+/ CD8+ T cells were isolated from splenocytes of untreated tumor-bearing mice, effector-target ratios from 1:1 to 50:1, then 100μl of each cell solution was seeded in triplicates into 96-well plate and incubated 48h. As for different groups, 3 hours before cells co-cultured, IFN-γ or perforin secretion was blocked by anti-IFN-γ (5 μg/ ml) or CMA (50 ng/ml) (Abdool et al., 2006) respectively, CPT was added at 10μM. Cell cytotoxic activity was detected by LDH-cytotoxic assay according to the protocol recommended by the manufacturer. Counted cell killed percentage (%) by using the formula: cytotixcity (%)= (test sample - low control)/ (high control-low control) ×100.

Western blot analysis

Following treatment, cells were washed with cold PBS on ice, then lysed in Cell lysis buffer for Western (Beyotime, China). Lysates were put on ice for 10 seconds

and centrifuged at 14, 000 rpm for 10 minutes at 4 °C. Protein concentration was determined by Enhanced BCA Protein Assay Kit with bovine serum albumin as standard (Beyotime). Equivalent amounts of protein were separated on 5% to 12% SDS- -polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were incubated with PBS containing 0.5% nonfat dry milk to block nonspecific binding and were incubated with primary antibodies, then with appropriate secondary antibodies conjugated to horse-radish peroxidase. Immunoreactive bands were visualized by using Pierce ECL Western Blotting Substrate (Thermo Scientific).

Statistical analysis

The data were analyzed using Prism 5.0 software (GraphPad). Experiments were repeated three times. The data presenting the differences between the groups were analyzed by one-way or two way ANOVA with Bonferroni post-test to compare tumor cvvolume in the treatment groups. $p<0.05$ indicated that the value of the test sample was significantly different from that of the relevant control.

Results

CPT inhibits MCF7 cells while stimulates splenocytes and CD4+ T cells proliferation

CPT has benzofuran structure (Figure 1a), this determines it has pharmacological properties. In order to test the antitumor efficiency of CPT, we firstly used different concentrations of CPT to treat MCF7 cells, as show in Figure 1b, treatment with CPT 48 hours significantly inhibited the growth of MCF7 cells in a concentration- -dependent manner. As we all know, the less cytotoxic effects in normal cells, the better for a drug, spleen as the main peripheral immune organ plays a vital role in antitumor (Watanabe et al., 1996; Klebanoff et al., 2004), so we also assessed the function of CPT on splenocytes and CD4+ T cells isolated from spleen of naive mice. Contrary to its effect on tumor cells, CPT stimulated CD4+ T cells proliferation under 10 μM, as for splenocytes, CPT still had positive function even in 20 μM (Figure 1b). Considered that CPT inhibited 40% MCF7 cells while stimulated 120% CD4+ T cells proliferation, so we chose 10 μM as experimental concentration.

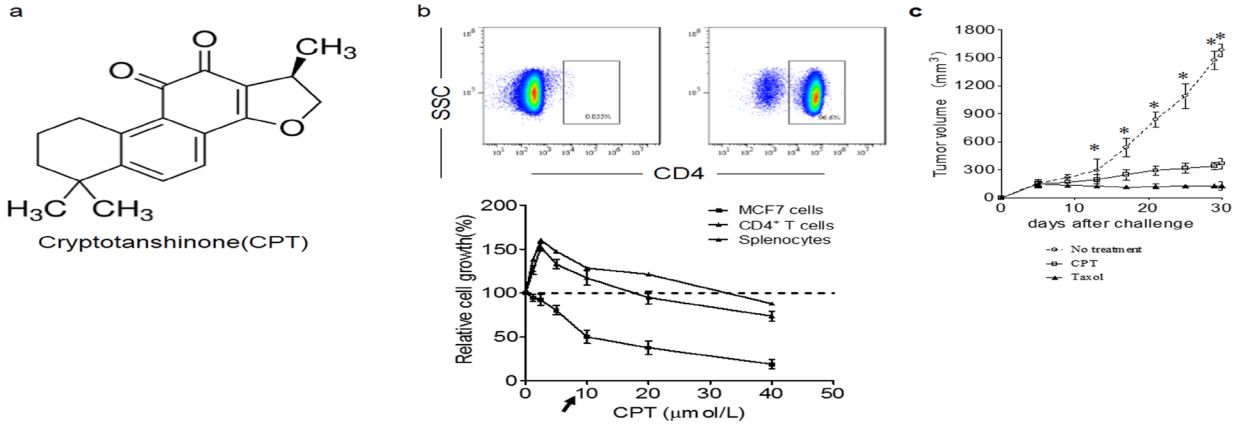

Figure 1. CPT inhibits the proliferation of MCF Cells *in vitro and in vivo*, but, on the Contrary, Positively Influences Normal Cells. (a) Chemical structure of CPT; **(b)** up showed the purity of magnetic isolation of CD4+ T cells; below, MCF7 cells grown in 96-well plates with DMEM supplemented with 10% FBS, were exposed to the indicated compounds (0-40 μM) for 48 h. Cell proliferation was evaluated using CCK-8 assay. **(c)** C57 mice were challenged with 1×10^6 MCF7 cells, then at day 5, tumor-bearing mice were treated with nothing(○), CPT(□) or Taxol(▲). Mean tumor volume were measured every 4 days.*$p<0.05$, No treatment versus CPT and Taxol. Data are representative of at least two independent experiments (n=6 mice per group). Error bars in C represent means±SD

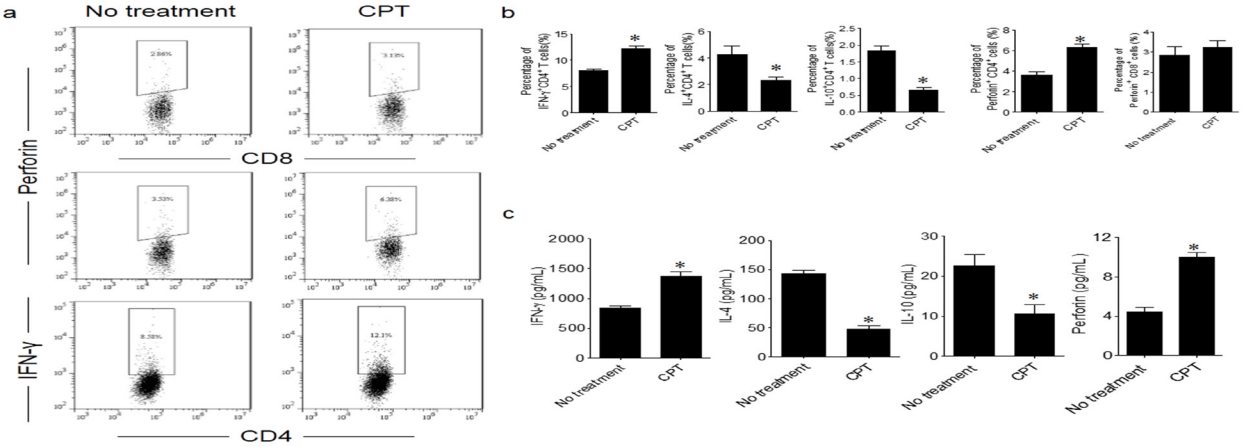

Figure 2. CPT Polarizes Immune Reaction Toward Th1 type and Increases Perforin Production of CD4+ T Cells. Tumor-bearing mice in Figure.1c were sacrificed at day 30, then detected perforin production in CD4+ and CD8+ T cells **(a)**. Other cytokines like IFN-γ, were measured in cellular level **(a, b)** or in serum**(c)**. *$p<0.05$, No treatment versus CPT, Error bars represent means±SD

CPT inhibits tumor growth in vivo

Similar with previous reports that CPT had antitumor function *in vitro* for many kinds of tumor cells (Park et al., 2013; Tse et al., 2013), our results also showed that CPT inhibited the proliferation of breast cancer cells (MCF7) *in vitro*. To further identify the antitumor activity of CPT *in vivo*, C57 mice all injected with 1×10^6 MCF7 cells, and then treated with CPT or Taxol since day 5, respectively. CPT significantly inhibited MCF7 cells growth from day 13 when compared with no-treatment mice, but still has a minor distance with the effect of taxol (Figure 1c). Considered that taxol is a typical drug to treat breast cancers, so this small gap of therapeutic effect between CPT and taxol can't deny that CPT has *in vivo* antitumor effect. Collectively, the data suggest that CPT may serve as an adjuvant for the treatment of breast cancers.

CPT enhances Type-1 immunity and perforin production in tumor-bearing mice

When talked about the mechanism that how CPT influences tumor cells growth, previous reports all focused on cell-cycle or apoptosis in the point of cell biology. We all know that immune system takes the responsibility to constrain or eradiate tumor, and Th1-dominant immunity is critically important for the induction of antitumor cellular immunity *in vivo* (Nishimura et al., 2000). Based on the fact that CPT has inhibited tumor growth *in vivo*, here, we investigated which T helper cytokines (Th1 or Th2) predominate in breast cancer upon treating with CPT. IL-4 and IFN-γ expressional level were detected in peripheral blood and spleen of tumor-bearing mice, as showed in Figure 2, CPT obviously elevated IFN-γ secretion while decreased IL-4 in serum, as well as in splenic CD4+ T cells. As for another Th2-tpye cytokine, IL-10, which is mainly produced by inducible (i) Treg or Tr1 cells present in cancer and mediate powerful suppression of effector T cells (Whiteside, 2012), to our surprise, suppression of IL-10+ CD4+ T cells (Tr1) was also observed in CPT-treated tumor-bearing mice, and also IL-10 level in serum (Figure 2b, 2c). Taking together of these results, we could conclude that CPT treatment resulted polarization of Th1-type immune response in breast cancer-bearing mice.

Other than cytokines-secreted CD4+ T cells, the key

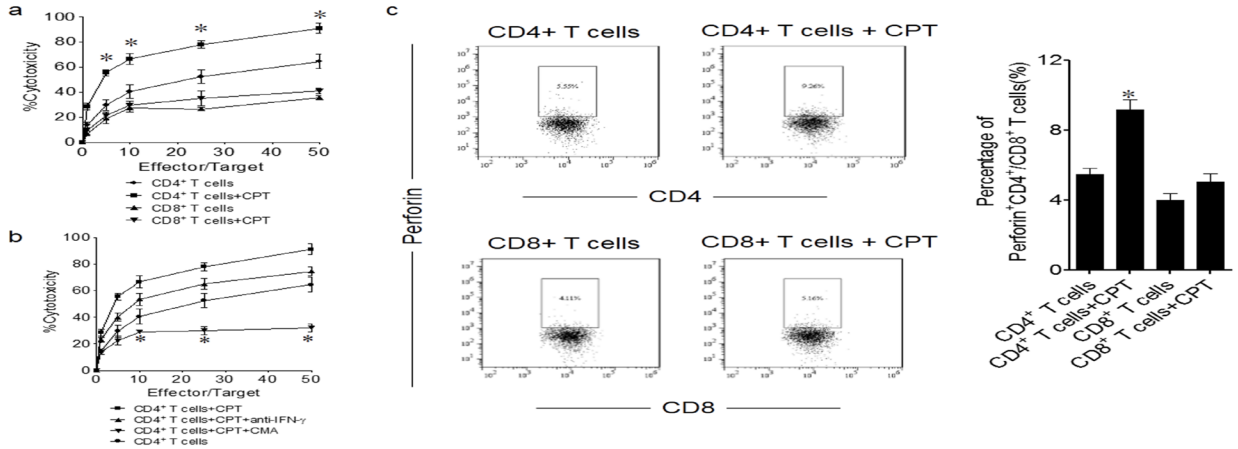

Figure 3. CPT Mainly Enhances Perforin-Mediated Cytotoxic Activity of CD4+ T Cells. CD4+ or CD8+ T cells were isolated from no-treatment tumor-bearing mice, and then incubated at different ratios with MCF7 cells for 48h; **(a)**. *p<0.05, CD4+ T cells+CPT versus other group. In vitro killing activity of CD4+ T cells was also tested in presence of 50 ng/ml concanamycin A (perforin inhibitor) or 5 μg/ml IFN-γ Ab; **(b)**. In vitro killing activity was determined by LDH-cytotoxic assay. perforin+ CD4/CD8 T cells in cytotoxic assay were quantified at the highest ratio 50:1; **(c)**. *p<0.05, CPT versus CPT-free group. Data are representative of three independent experiments. Error bars represent means ± SD

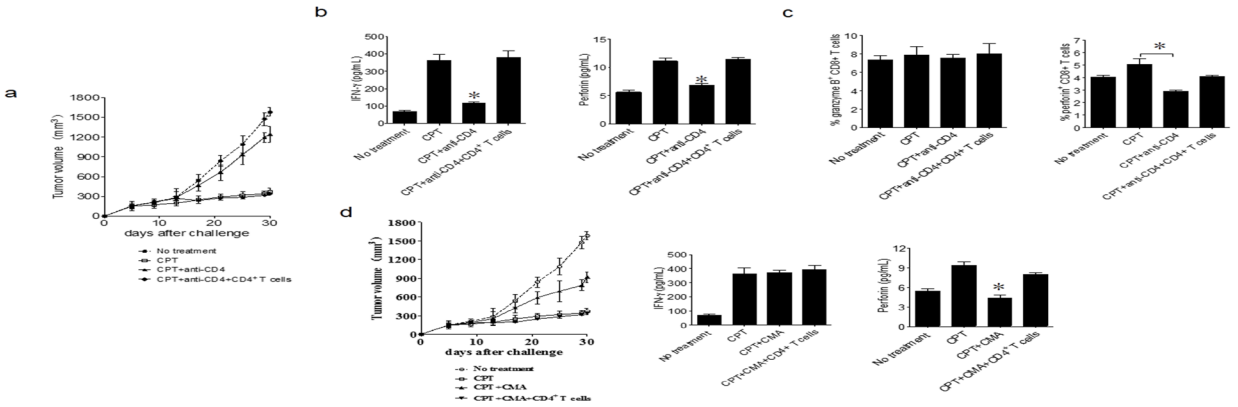

Figure 4. CPT depends on Cytotoxic CD4+ T Cells to Inhibit *in vivo* Tumor Growth. Tumor-bearing mice were treated with anti-CD4 **(a)** or CMA **(d)** two days before tumor cells injection, CD4+ T cells from cognate tumor-bearing mice adoptively transferred into recipient at the same day with tumor cells inoculation. Tumor volume measured same as Figure. 1C. Mice were sacrificed at day 30, and then detected the level of cytokines in serum **(b and d)** or cellular cytokines production in splenocytes **(c)**. Error bars represent means±SD. *p<0.05, CPT+anti-CD4/CMA versus CPT and CPT+anti-CD4/CMA+CD4+ T cells group

immune cells responsible for antitumor activity are the CTLs (Aerts et al., 2013). Besides CD8+ cytotoxic T cells, an increasing number of studies have found that Cytotoxic CD4+ T cells in malignancies (Fu et al., 2013). In CPT-treated tumor-bearing mice, the percentage of Cytotoxic CD4+ T cells significantly higher than no-treatment group, but there was no significant difference for CD8+ cytotoxic T cells (Figure 2a, 2b), peripheral perforin level showed the same change with Cytotoxic CD4+ T cells (Figure 2). These results indicate that CPT possibly through CD4+ T cells to exert its antitumor activity.

CPT exploits Cytotoxic CD4+ T cells to kill tumor cells in vitro

CPT-treated mice has a higher level of Cytotoxic CD4+ T cells, next, we performed cell cytotoxic assay to confirm whether CPT could enhance CD4+ T cells cytotoxic activity. CD4+ and CD8+ T cells freshly isolated from No-treatment tumor-bearing mice, and then co-cultured with different ratio of MCF7 cells. As show in Figure 3a, CPT markedly increased the cytotoxicity of CD4+ T cells. Similar phenomenon also occurred in flow cytometry analysis that the percentage of perforin+ Cytotoxic CD4+ T cells was raised by CPT (Figure 3c). As for CD8+ T cells, *in vitro* results further identified that CPT has insignificant influence on CD8+ T cells. CD4+ T cells could change tumor outcome by secreting IFN-γ or perforin, and previous results (Figure 2) have showed that these two cytokines all be promoted by CPT, thus, we blocked IFN-γ or inhibited perforin-mediated cytotoxicity by CMA to figure out how CPT influences the antitumor activity of CD4+ T cells. In Figure 3b, CMA not only offset the cytotoxic activity of CD4+ T cells increased by CPT, and also autogenic cytotoxic activity induced by tumor. Therefore, perforin is the main cytokine exploited by CD4+ T cells to lyse MCF7 cells, and CD4+ cytotoxic T cell is vital to the antitumor effect of CPT.

CPT mainly depends on perforin secreted by Cytotoxic CD4+ T cells to inhibit tumor growth in vivo

To ensure CD4+ T cells play central role in CPT-induced tumor inhibition, before treated with CPT, CD4+ T cells was depleted in tumor-bearing mice, when compared with CPT-treated group, it was found that CPT lost its antitumor growth activity with the depletion of CD4+ T cells, tumor growth curve even similar with no-treatment group (Figure 4a). After injected congeneric naive CD4+ T cells into CD4+ T cells-depleted mice, CPT regenerated its antitumor activity. We detected perforin level in serum of tumor-bearing mice, after depleted CD4+ T cells, peripheral perforin significantly decreased (Figure 4b), perforin+CD8+ cytotoxic T cells in spleen also dramatically decreased with CD4+ T cell depletion (Figure 4c), this may own to the loss of assistance provided by CD4+ T helper cells. Finally, to test the direct perforin+ CD4+ T cells dependence of *in vivo* suppression to breast tumor, perforin-mediated cytotoxicity was revoked by CMA, tumor growth was significantly accelerated in CPT-treated tumor-bearing mice, however, when additionally transferred CD4+ T cells into CMA-injected tumor-bearing mice, tumor growth has no obvious difference

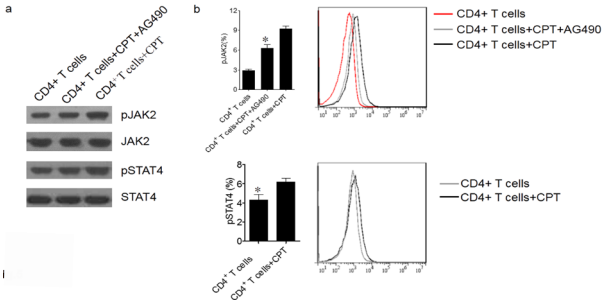

Figure 5. JAK2/STAT4 Pathway Plays a Role in CPT-Mediated Perforin Production of CD4+ T Cells. CD4+ T cells from tumor-bearing mice treated with CPT and/or 50μM AG490 for 48 hours, then detected JAK2 and STAT4 phosphorylation by Western blot with indicated antibodies **(a)** or FCM **(b)**. *p<0.05, *labeled group versus other group

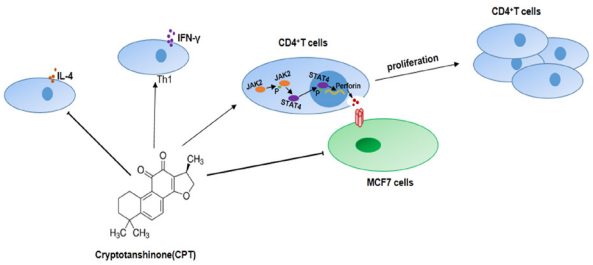

Figure 6. A model of CPT Induces Cytotoxic CD4+ T Cells through JAK2/STAT4 Pathway to Inhibit Breast Tumor Growth

with CPT-treated group (Figure 4d), perforin level in serum also rebounded from CMA-treatment. The data suggest that perforin secreted by transferred CD4+ T cells counteracts the effect of CMA and is important for CPT to exert its anti-breast cancer activity.

CPT through JAK2/STAT4 pathway to activate CD4+ T cells

In the recent ten years, numerous articles have demonstrated that JAK/STAT pathway play a crucial in carcinogenesis (Yoshikawa et al., 2001; Calvisi et al., 2006; Zhuang et al., 2012) and lymphocytes activation (Tamiya et al., 2011), evidence also revealed that human perforin gene is a direct target of STAT4, IL-12 induces cytotoxicity of NKT cells through JAK2/STAT4 pathway (Yamamoto et al., 2002). In our experiments, Data have showed that CPT stimulates CD4+ T cells to secrete perforin, so we questioned the possibility that CPT may act like IL-12 to activate CD4+ T cells. We detected pSTAT4 and pJAK2 level in CPT-treated CD4+ T cells, fortunately, we found that CPT significantly enhanced JAK2 and STAT4 phosphorylation, and this phenomenon can be reversed by AG490, a JAK inhibitor. So we demonstrated that CPT acts like IL-12 to induce CD4+ T cells secrete perforin, and then perform cytotoxicity target MCF7 cells.

Discussion

Most recently, data have shown that CPT is the most potent anticancer agent among the extracts of Danshen, by inhibiting proliferation of cancer cells or inducing cell apoptosis (Chen et al., 2010; Chen et al., 2012), but its function on MCF-7 cells still rarely reported. The

human breast cancer cell line , MCF7, is ER-positive, approximately two-third of breast cancers are ER-positive (Nizamutdinova et al., 2008), therefore, it is important to identify new agents that is effective in breast cancers. In this report, CPT powerfully inhibited the growth of MCF7 cells in a dose-dependent manner, similar with Gong Yi's observation (Gong et al., 2012). Animal study also showed that CPT significantly inhibits the growth of breast cancer cells *in vivo*, just slightly weaker than taxol. This is the first report, to the best of our knowledge, that CPT had potent anti-breast cancer activity in both *in vivo* and *in vitro* assays. As a drug, its side effects cannot be ignored, so we measured proliferative effect of CPT on normal cells, a recent study have shown that CPT at 50 μM did not exhibit significant cytotoxicity in normal cells (Gong et al., 2011), in this study, we also found that CPT just showed mild cytotoxic effect in splenocytes until at quite high concentrations (>40 μM). Less than 20 μM, CPT even had positive effect both on CD4+ T cells and splenocytes. This further highlights the potency of CPT for tumor treatment in the future.

To gain insight into the mechanism of CPT to perform its function, we initially analyzed multiple immune parameters; results demonstrated that CPT polarized immune reaction toward Th1 type, significantly stimulated IFN-γ production of CD4+ T cells. Considered that tumor-reactive Cytotoxic CD4+ T cells have been a bright point in immunotherapy of tumor (Porakishvili et al., 2001; Quezada et al., 2010; Akhmetzyanova et al., 2013), so we also measured perforin production of CD8+ T cells as well as CD4+ T cells. Unfortunately, CPT showed marginal effect on CD8+ cytotoxic T cells, even though that CD8+ cytotoxic T cells are classic tumor killing cells (Ohmura et al., 2008), but enhanced perforin production of CD4+ T cells. To further verify this result, we isolated CD4+ and CD8+ T cells from tumor-bearing mice and analyzed their cytotoxic ability, results same with *in vivo* data. Up to now, we knew that CPT increased IFN-γ and perforin production of CD4+ T cells simultaneously, the question is that which cytokine CPT dependent to exhibit antitumor function or both. In the following experiments, Blocked IFN-γ or perforin production revealed that CMA dramatically decreased cytotoxic activity of CD4+ T cells, but not IFN-γ Ab. In vivo tumor challenge experiment further verified that Cytotoxic CD4+ T cells work as a tool for CPT to restrain tumor growth, because CMA treatment or depletion of CD4+ T cells all significantly reversed anti-breast cancer activity of CPT. Why CPT selectively depend on CD4+ T cells but not CD8+ T cells and what's role IFN-γ played in CPT-mediated protective effect remains still need to be figure out in future.

Despite previous findings have introduced many signal pathways that CPT employed to perform anti-cancer activities, the molecular mechanism about how CPT increased perforin production in CD4+ T cells remain elusive. According to Pierre Gosselin's report, perforin promoter has a STAT binding site and is regulated by different types of STAT protein (Yu et al., 1999); K Yamamoto also found that IL-12 could bind to IL-12R and then activate JAK2-STAT4 pathway to trigger STAT4 binding to perforin promoter, and so resulted the expression of perforin (Yamamoto et al., 2002). Based on these previous fruits, we detected JAK2 and STAT4 phosphorylation level in CD4+ T cells upon CDT stimulating. Our data revealed that CPT activated JAK2-STAT4 pathway just like IL-12. Although we found that CPT work like IL-12, there still may have other pathways for CPT to increase perforin production and should be the important area of research in the future.

In conclusion, our work firstly identified the antitumor potency of CPT *in vivo*, as CPT markedly increased the cytotoxic ability of CD4+ T cells and so restricted tumor growth. In vitro analysis also showed that CPT works like IL-12 through JAK2/STAT4 pathway to activate CD4+ T cells (Figure 6). Finally, we believe that these data provide promising experimental evidence to support that CPT may be a novel efficacious and safe candidate agent for the therapy of breast cancer progression by induction of Cytotoxic CD4+ T cells and inhibition of proliferation of breast cancer cells, Our results also provided functional evidence to support that Cytotoxic CD4+ T cells are efficient target to treat tumors.

Acknowledgements

The authors thank members of their laboratory and their collaborators for their research work, in particular professor Cheng-liang Zhu, Department of Clinical Laboratory, Renmin Hospital of Wuhan University, Wuhan, P.R. China

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