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PRINCIPAL INVESTIGATOR: Jin-Rong Zhou

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

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| 14. ABSTRACT The purposes of this project were to evaluate if the tanshinone I (T1) and tanshinone IIA (T2A) combinations have synergistic effects on the growth of prostate tumors and to elucidate the cellular and molecular mechanisms of actions. The rationale of proposing synergistic combination of T1 and T2A was based on our preliminary data showing that T1 and T2A had the potent anti-angiogenesis and anti-prostate cancer growth activities, respectively. The results from this research project showed that T1 and T2A alone showed significant effects on inhibiting the growth of androgen-independent PC3 tumors, and that T1 and CT, but not T2A, showed significant effects on inhibiting the growth of androgen-dependent LNCaP tumor. Tanshinones showed minimal side effect on food intake or body weight. On the other hand, the proposed T1 and T2A combination did not show a synergistic or an additive effect, but rather an antagonistic effect on prostate tumor growth in vivo. This unexpected combination effect was supported by the later in vitro studies showing that the T1 and T2A combination indeed had a suggested antagonistic effect on prostate cancer cell growth. The in vitro studies also strongly suggest that the CT and T1 combination, or the CT and T2A combination may have synergistic effect on prostate cancer cell growth. Mechanism studies defined Aurora A (and also possibly Aurora B) and SIRT1 may be important functional molecular targets for tanshinones actions. Our research supports tanshinones, especially T1, as efficacious and safe agents for prostate cancer prevention and therapy, and suggests that tanshinones may be a novel group of candidate agents as Aurora kinases inhibitors. Our research also strongly suggest the rationale to future investigate the synergistic effect of T1 and CT combination regimen for effective prevention and therapy of prostate cancer. | | | | | | |
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INTRODUCTION

The search for effective regimens with minimal adverse effects for the treatment of prostate cancer remains the top priority of prostate cancer research. A Chinese herb, Danshen has been widely adopted in the traditional Chinese medicinal preparations for treatment of cerebrovascular disorders with minimal side effects. Our preliminary *in vitro* studies showed that tanshinones IIA (T2A) had potent activity in inhibiting the growth of prostate cancer cells in part via induction of apoptosis, and tanshinone I (T1) had potent anti-angiogenesis activity. Prostate tumorigenesis involves multiple critical steps, such as uncontrolled cancer cell growth and increased tumor angiogenesis. Considerable data indicate that the combination of agents that target different pathways may have a synergistic effect. In this proposal, we hypothesize that T1 and/or T2A may serve as effective therapeutic agents with minimal adverse effects for prostate cancer, and the combination of T1 and T2A may have a synergistic effect on prostate cancer therapy. The objectives of this proposal are to define the therapeutic efficacy of T1 and T2A, alone and in combinations on prostate cancer progression in clinically relevant animal models of prostate tumor progression, and to identify the cellular and molecular biomarkers associated with therapeutic activities of T1 and T2A to gain insights into possible mechanisms of action. Specific Aim 1 is to determine the effects of T1 and T2A, alone and in combinations on the growth and metastasis of both androgen-dependent and androgen-independent human prostate tumors in animal models. Specific aim 2 is to determine the effects of T1 and T2A, alone and in combinations on delaying hormone ablation-induced prostate tumor progression to androgen-independent prostate tumors. Specific aim 3 is to identify the *in vivo* biomarkers that are associated with the activities of T1 and T2A.

BODY

The proposed experiments in all three specific aims have been finished. The followings are detailed experiments in each specific aim and results.

1. Specific aim 1: to determine the effects of T1 and T2A, alone and in combinations on the growth and metastasis of both androgen-dependent and androgen-independent human prostate tumors in animal models (Tasks 1 and 2)

This aim consists originally of two animal studies to evaluate the efficacy of T1 and T2A, alone and in combinations, on the growth and progression of androgen-dependent LNCaP and androgen-independent PC-3 prostate tumors and to determine the modulation of associated cellular and molecular biomarkers. As stated in the previous progress report, we made the following modifications on the experiments: (1) Instead of conducting firstly the animal study in LNCaP tumor model (original SOW for the first year), we decided to apply the androgen-independent PC-3 tumor model to evaluate the effects of T1 and T2A on the growth and metastasis of androgen-independent prostate tumors (original SOW for the second year) because PC-3 tumors are easier to establish in mice than the LNCaP tumors and thus it is easier to use the PC-3 tumor model to establish the experimental conditions (such as the dose response, the route of administration, etc). (2) Since it is unclear the efficacious doses of the compounds for significant growth inhibition, we decided to determine the dose-dependent responses of the PC-3 tumors to the treatments of T1 and T2A.

1.1. Animal studies to determine the experimental conditions (the dose-dependent response and the route of administration) in PC-3 tumor animal model (revised pilot studies)

Briefly, male SCID mice (eight-week-old) were purchased from Taconic (Germantown, NY), fed the AIN-93M diet for one week of adaptation, and randomized into seven experimental groups (n=8/group). Mice were then be inoculated intraprostatically with 2×10^6 PC-3 cells, and treated with the following experimental treatments: (1) Control: vehicle only; (2) and (3): T1 at 20 and 40 mg/kg BW; (4)-(5): T2A at 20 and 40 mg/kg BW. The compounds were suspended in 0.5% CMC, and then administered via oral gavage. Food intake and body weight were measured weekly. At 4 and 8 weeks after tumor cell inoculation, phlebotomy was performed by accessing the retro-orbital venous plexus to obtain 100 μ L blood, and the blood level of IL-6 was measured at different time points to monitor tumor development and growth. The experiment was finished when the average tumor weights in the control animals reached about 2% of the body weight (about 8-9 weeks). The mice were sacrificed; primary tumors were excised and weighed. A tumor slice from each primary tumor tissue was carefully dissected and fixed in 10% buffer-neutralized formalin, paraffin-embedded, and sectioned at 4 μ m thickness for histology and immunohistochemistry. An aliquot of tumor samples was used for total RNA extraction for cDNA microarray assays. Lymph nodes and lungs were harvested, fixed, paraffin-embedded and H&E stained for metastases quantification.

Table 1. Effects of T1 and T2A on the growth and metastasis of PC-3 tumors

| Treatment | Final tumor weight (g) | Lymph node metastases (#/mouse) |
|------------------|------------------------------|---------------------------------|
| Control | 1.26 \pm 0.27 ^a | 2.8 \pm 0.5 ^a |
| T1 (20mg/kg BW) | 1.27 \pm 0.20 ^a | 2.3 \pm 0.6 ^a |
| T1 (40mg/kg BW) | 1.13 \pm 0.20 ^a | 3.1 \pm 0.8 ^a |
| T2A (20mg/kg BW) | 1.14 \pm 0.16 ^a | 2.9 \pm 0.4 ^a |
| T2A (40mg/kg BW) | 0.92 \pm 0.23 ^a | 2.1 \pm 0.5 ^a |

Values are means \pm SEM. Within the column, values with different superscript letters are significantly different with $p < 0.05$.

As shown in Table 1, the T1 or T2A at the proposed doses did not significantly inhibit the growth of PC-3 tumors although the dose-dependent responses were indicated. The results suggest that the doses we proposed may not be high enough to exert the significant inhibitory effects on the growth of PC-3 tumors. The following factors may contribute to the non-significant effects: (1) the poor bioavailability of T1 and T2A in mice due to their limited solubility and once/day oral gavage; (2) T1 or T2A may have less potent activity in inhibiting the growth/progression of PC-3 tumors than that of LNCaP tumors. In the meantime, we conducted the in vitro studies and confirmed the potent activities of T1 and T2A in inhibiting angiogenesis and the growth of prostate cancer cells, respectively. Thus we believe that the non-significant in vivo activity of T1 or T2A is in part due to the poor bioavailability.

In other studies, we found that oral gavage of T1 and T2A at 200mg/kg BW did not significantly alter food intake or body weight, suggesting minimal side effects of these compounds. Therefore, we conducted another animal study to further determine the efficacious doses of T1 and T2A at the higher doses (100 and 200mg/kg BW) and the routes of administration (oral gavaging with corn oil or dietary supplementation) in inhibiting the growth of PC-3 tumors. We found that T1 and T2A at the proposed doses (100 and 200 mg/kg BW) and the route of administration (oral gavaging with corn oil) showed growth inhibition activity against PC3 tumors. Although dietary supplementation was labor-efficient, the intake of the active agents could not be controlled because the food intake couldn't be precisely controlled. By comparing the data, we concluded that the oral gavaging with corn oil the vehivle could increase

the bioavailability of the agents and the doses could be well controlled. Therefore, in the subsequent animal experiments, we used oral gavage method. In analyzing the dose-dependent responses, we found that mice at these doses did not change food intake or body weight, confirming low adverse effects of these agents at the given doses.

1.2. The animal study to determine the effect of T1 and T2A, alone and in combination, on the growth of PC-3 tumors (revised task 1, original task 2)

In the PC-3 tumor animal study, we followed the animal protocol used in the pilot studies. Briefly, male SCID mice (eight-week-old) were fed the AIN-93M diet for one week of adaptation, and randomized into ten experimental groups (n=8/group). Mice were then be inoculated sc with 2×10^6 PC-3 cells, and treated with the following experimental treatments: (1) Control: vehicle (100 μ L corn oil) only; (2) and (4): T1 at 37.5, 75 and 150 mg/kg BW, respectively; (5)-(7): T2A at 37.5, 75 and 150 mg/kg BW, respectively; (8) and (10): T1 and T2A combination at 37.5/37.5, 75/75 and 150/150 mg/kg BW, respectively. The compounds were suspended in corn oil and each mouse was given the same amount of corn oil so that the caloric intake from corn oil was the same. Tumor volume, food intake and body weight were measured weekly. At the end of experiment, the mice were sacrificed, and primary tumors were excised and weighed. A tumor slice from each primary tumor tissue was carefully dissected and fixed in 10% buffer-neutralized formalin, paraffin-embedded, and sectioned at 4 μ m thickness for histology and immunohistochemistry. An aliquot of tumor samples was used for total RNA extraction for future analysis. Lymph nodes and lungs were harvested, fixed, paraffin-embedded and H&E stained for metastases quantification.

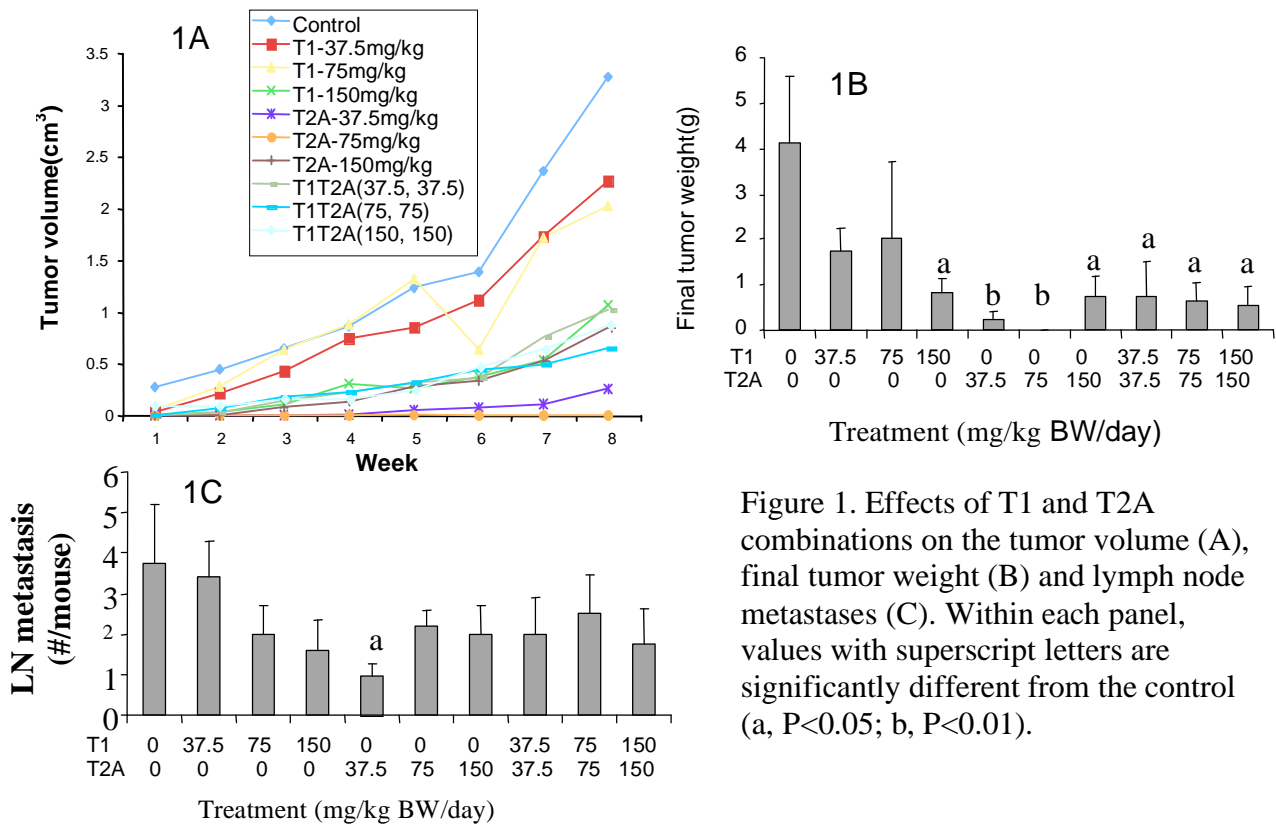


Figure 1. Effects of T1 and T2A combinations on the tumor volume (A), final tumor weight (B) and lymph node metastases (C). Within each panel, values with superscript letters are significantly different from the control (a, P<0.05; b, P<0.01).

In addition to the PC-3 study, we also performed the similar animal study using another androgen-independent prostate tumor model, DU145, to evaluate the effect of T1 on DU145

tumor growth since previous in vitro studies supported that T1 was the most potent tanshinone for prostate cancer inhibition in vitro. We used T1 at 150mg/kg BW via gavaging daily. The experimental design and protocols are provided in the published paper (appendix 1).

The results of both androgen-independent prostate tumor models are summarized as follows.

1) Food intake and body weight did not change among the experimental groups. In fact, food intakes in the treatment groups were slightly increased, compared with the control. It suggests that the agents are well consumed by the animals and have minimal adverse effects on animal health.

2) Figure 1A shows the time-dependent effects of treatments on the tumor growth, and the figure 1B shows the final tumor weight. Although all treatments inhibited the growth of PC-3 tumors, the effects of T2A groups were more potent than that in the T1 groups. Due to potent activity of T2A groups, we were unable to determine the nature of combination effect between T1 and T2A. Instead, the results suggest the combination may be neither synergistic, nor additive. Further studies are needed to determine the combination effect.

3) The treatments also inhibited metastases of PC-3 tumors to the lungs or lymph nodes (1C). On the other hand, the anti-LN metastasis activities of the compounds were not as potent as the anti-tumor growth activities. Again, the combinations of T1 and T2A did not show additive or synergistic effects.

4) The T1 treatment also significantly inhibited the growth of DU145 tumors in vivo (Fig 5 in appendix 1), but did not significantly inhibit metastasis (data not shown).

5) In summary, although the results did not verify the synergistic effect of T1 and T2A combinations on the growth and metastasis of PC-3 tumors, we determined the potent activity of individual agents in growth inhibition of PC-3 and DU145 tumors.

1.3. The animal study to determine the effect of T1 and T2A, alone and in combination, on the growth of LNCaP tumors (revised task 2, original task 1)

Similarly, we also conducted the LNCaP tumor animal study to determine the effects of T1 and T2A, alone and in combinations on the growth of androgen-sensitive prostate tumors. Since cryptotanshinone (CT) is another major tanshinone in Danshen, we also evaluated its effect on the growth inhibition of LNCaP tumors so that the anti-LNCaP activities of all three major tanshinones could be compared simultaneously. In brief, male SCID mice were fed the AIN-93M diet for one week of adaptation before being inoculated orthotopically with 2×10^6 of LNCaP cells. Mice were randomly assigned into one of the following experimental groups (n=12/group) and treated with the assigned experimental treatments: (1) Control (100 μ l corn oil as vehicle), (2)-(3) T1 in corn oil at 100mg/kg BW and 200 mg/kg BW by gavage daily, (4)-(5) T2A in corn oil at 100mg/kg BW and 200 mg/kg BW by gavage daily, (6)-(7) CT in corn oil at 100mg/kg BW and 200 mg/kg BW by gavage daily, (8)-(9) T1/T2A combinations at 100/100 and 200/200 mg/kg BW. Food intake and body weight were measured weekly. At 4 and 8 weeks after tumor cell inoculation, phlebotomy was performed by accessing the retro-orbital venous plexus to obtain 100 μ L blood from each animal, and serum PSA levels were measured by ELISA assay to monitor tumorigenesis rate and tumor size. At week 6 and 8, micro-CT was applied to visualize tumor development and growth. At the end of the experiments (12 weeks after cell inoculation), the mice were sacrificed; primary tumors were excised and weighed. A tumor slice from each primary tumor tissue was carefully dissected and fixed in 10% buffer-neutralized formalin, paraffin-embedded, and sectioned at 4 μ m thickness for immunohistochemistry. An aliquot of each tumor sample was used to extract total RNA for real-time PCR. The results are shown as

follows.

1) As shown in Figure 2A, both T1 and CT showed the dose-dependent effect on inhibiting the growth of LNCaP tumors, but to our surprise, T2A did not show significant effect. This finding of T2A activity was contrast to that in the PC-3 tumor model. It is unclear at this time why T2A showed so different activities in these two tumor models since the in vitro studies indicated similar activities of T2A in inhibiting the growth of these two cell lines.

2) Interestingly, CT also showed significant activity. Our in vitro studies showed CT had the least potent activity against prostate cancer cell growth with IC50s around 25-50 μM (Figure 1 in appendix 1), compared with T1 or T2A. This increased bioactivity of CT may be in part due to its higher bioavailability since it was reported that CT had higher bioavailability than other tanshinones. Indeed, we noted the darker color of urine from mice treated with CT than that with T1 or T2A, suggesting higher bioavailability of CT in vivo. This interesting observation should be further confirmed by measuring urinary and tissue metabolites in animals treated with different tanshinones. However, the lack of T2A activity can't be explained by its bioavailability since T2A was also bioavailable in vivo. It is speculated that specific metabolites of T2A may be responsible for the loss of T2A bioactivity in vivo.

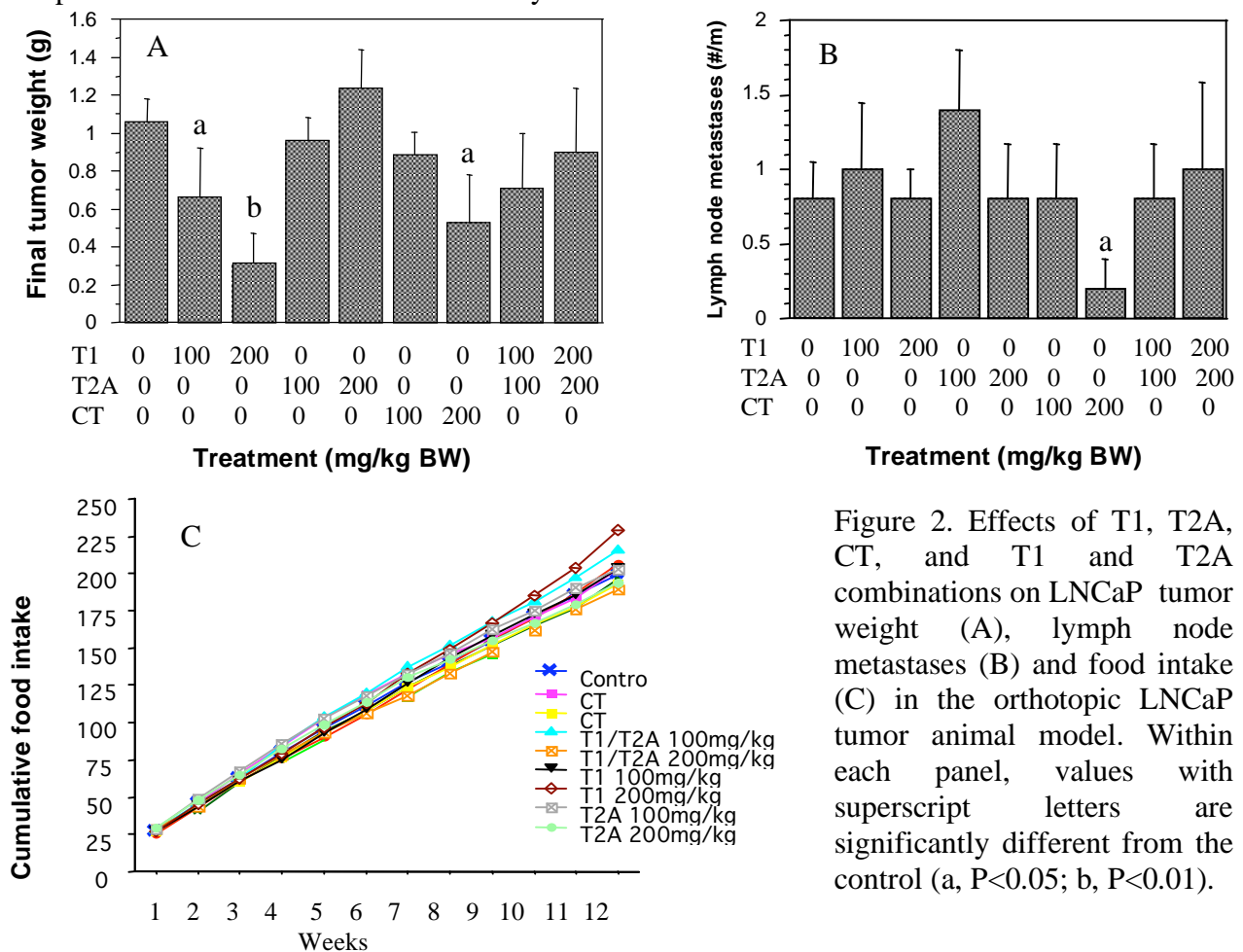


Figure 2. Effects of T1, T2A, CT, and T1 and T2A combinations on LNCaP tumor weight (A), lymph node metastases (B) and food intake (C) in the orthotopic LNCaP tumor animal model. Within each panel, values with superscript letters are significantly different from the control (a, $P < 0.05$; b, $P < 0.01$).

3) The combination of T1 and T2A did not show further enhanced activity in inhibiting the LNCaP tumor growth, suggesting that the combination may not be synergistic or additive. This finding is consistent with that in PC3 model. **These findings suggest that the combinations of tanshinones may have antagonistic effects on inhibiting the growth of prostate tumors.**

4) Both T1 and T2A did not show significant effect on inhibiting LN metastasis (Fig 2B),

but CT inhibited LN metastasis in a dose-dependent manner and the inhibitory effect at 200mg/kg BW was significant (Fig 2B). This finding suggests that CT may have potent anti-growth and anti-metastasis activities against LNCaP tumor in vivo.

5) The treatments did not significantly alter food intake (Fig. 2C) or body weight (data not shown), confirming that tanshinones at the efficacious doses do not have significant side effects.

6) The inhibitory effects of CT and T1 on LNCaP tumor growth were associated with significant induction of LNCaP cancer cell apoptosis, reduction of cancer cell proliferation, and inhibition of tumor angiogenesis (Figure 3).

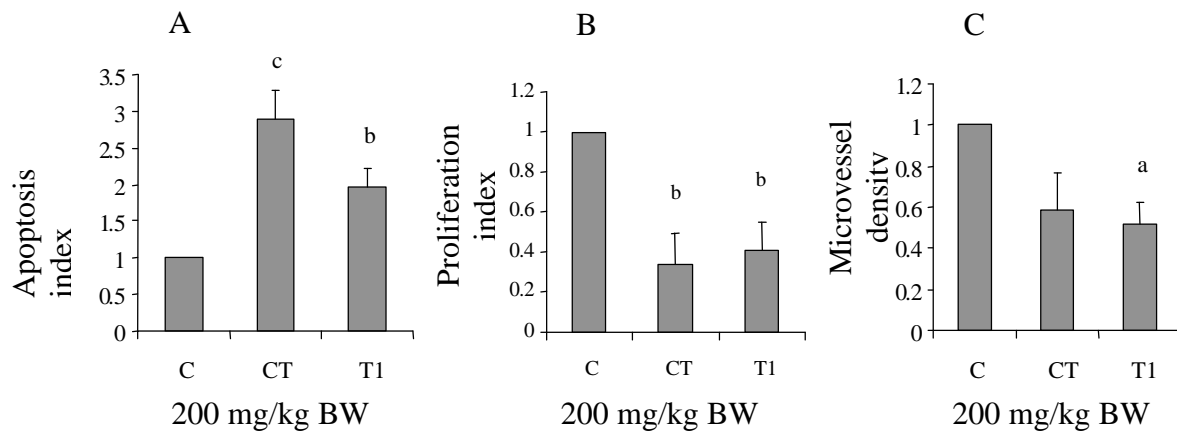


Figure 3. Effects of T1 and CT at 200mg/kg BW on LNCaP tumor cell apoptosis (A) and proliferation (B), and LNCaP tumor microvessel density (C). Within panels, values with a letter are significantly different from the respective controls (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$).

1.4. Additional in vitro studies to determine the tanshinone combination effect on prostate cancer cells (additional experiments directly related to tasks 1 and 2)

Our original hypothesis was based on our preliminary findings that T1 and T2A were potent candidate agents to inhibit angiogenesis and prostate cancer cell proliferation, respectively, thus the T1 and T2A combination may have synergistic effect on prostate cancer therapy by targeting prostate cancer cell growth and angiogenesis simultaneously. However, our above animal studies (and the animal study in specific aim 2 below) did not support a synergistic combination effect between T1 and T2A on prostate cancer treatment, but instead suggesting an antagonistic combination effect. We therefore applied the in vitro system to further determine the nature of tanshinone combination effect on prostate cancer cell growth. Our studies showed that the CT and T1 combination or the CT and T2A combination inhibited the growth of prostate cancer cells in a synergistic manner, whereas the T1 and T2A combination did not have synergistic effect on growth inhibition. The representative results using LNCaP cell line are shown in Figure 4. After initial experiments suggesting synergistic combinations between CT and T1 (Fig 4A), or CT and T2A (4B), we further performed experiments using the combination doses designed for synergy calculation, and the synergistic combination was verified by isobolograms (Fig 4C, 4D). The combinations between CT and T1 (Fig 4E), or between CT and T2A (Fig 4F) also further induced apoptosis of LNCaP cells, and the calculation of the combination indices support synergistic effects of the combinations on apoptosis induction ($CI < 1.0$, results not shown). These results suggest that the combination of CT with T1 or T2A may

have a synergistic effect on inhibiting the growth of prostate cancer cells in part by inducing apoptosis synergistically. These results also suggest that the T1 and T2A combination, which was the one we proposed to evaluate, may not have a synergistic effect on prostate cancer growth in vivo, despite that T1 and T2A were potent agents to target angiogenesis and prostate cancer growth, respectively, in vitro.

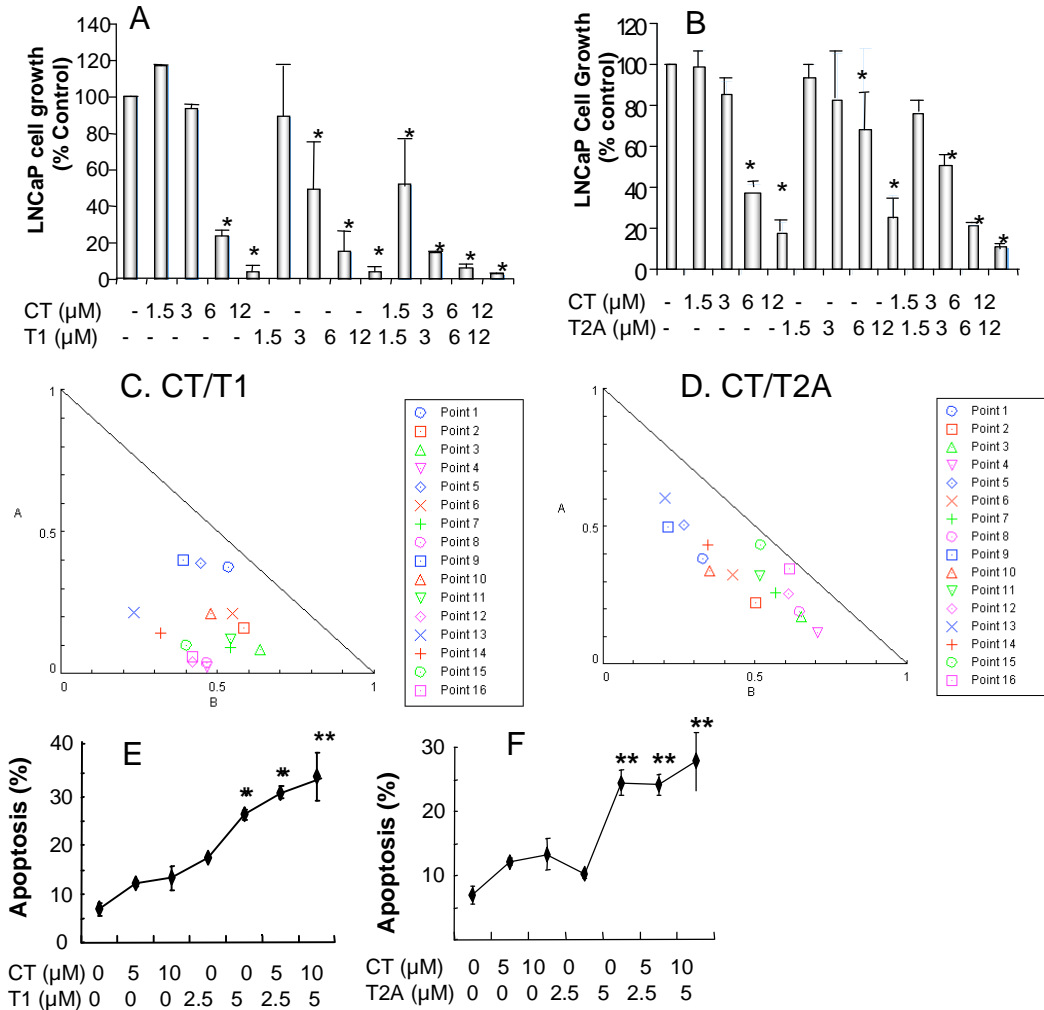


Figure 4. Effects of CT/T1 combinations (A) and CT/T2A combinations (B) on the growth of LNCaP prostate cancer cell line, and the calculated isobolograms showing the synergistic CT/T1 combination (C) and the synergistic CT/T2A combination (D). CT/T1 combinations (E) and CT/T2A combinations also showed synergistic effects on inducing apoptosis of LNCaP cell line. In each panel, values with “*” are significantly different from the control (* P<0.05, ** P<0.005)

2. Specific aim 2: To determine the effects of T1 and T2A, alone and in combinations on delaying hormone ablation-induced prostate tumor progression to androgen-independent prostate tumors (Task 3)

The objective of the studies in specific aim 2 was to evaluate the combined effects of T1 and T2A on delaying the androgen ablation-induced progression to hormonal independent/refractory phenotype in our well-established orthotopic prostate tumor animal model. In brief, after acclimation, male SCID mice were implanted orthotopically with LNCaP cells and consumed the AIN-93 control diet. Food intake and body were monitored weekly. At 5-6 weeks after tumor cell inoculation, phlebotomy was performed to obtain 100 μ L of blood from each animal, and serum PSA level was measured by ELISA. In addition, micro-CT was used to visualize tumor development and size. Mice with tumors were castrated and randomly assigned into seven experimental groups, and receive the following experimental treatments: (1) control (corn oil as vehicle, oral gavaging), (2)-(4) T1 at 50, 100 and 200 mg/kg BW, respectively, via oral gavaging, (5)-(7) T2A at 50, 100 and 200 mg/kg BW, respectively, (8)-(10) T1/T2A combinations at 50/50, 100/100 and 200/200 mg/kg BW, respectively. At several time points, blood samples were collected and measured for PSA. In addition, the growth of prostate tumor was monitored by using the micro-CT. The experiment was finished 15 weeks after cell implantation. At the end of experiment, the mice were sacrificed; primary tumors were excised and weighed. A tumor slice from each primary tumor tissue was carefully dissected and fixed in 10% buffer-neutralized formalin, paraffin-embedded, and sectioned at 4 μ m thickness for histology and immunohistochemistry. An aliquot of each tumor sample was used to extract total RNA for real-time PCR. Lymph nodes and lungs were harvested, fixed, paraffin-embedded and H&E stained for metastases quantification.

The results showed that:

1) As shown in Figure 5, T1 showed a dose-dependent effect on delaying the progression of castration-induced progression of androgen-independent prostate tumor, but T2A showed limited activity.

2) The combinations of T1 and T2A did not show significant additive or synergistic activity in delaying prostate cancer progression, consistent with our previous findings.

3) No treatments significantly inhibited lymph node or lung metastases (data not shown).

4) The data also confirmed limited side effect of T1 or T2A treatment in vivo.

3. Specific aim 3: To identify the *in vivo* biomarkers that associate with the activities of T1 and T2A (Tasks 1, 2 and 3)

The objectives of this specific aim are to determine the effects of tanshinone treatments on the modulation of cellular and molecular biomarkers in tumor samples from the above in vivo efficacy studies to understand the underlying cellular and molecular mechanisms of tanshinones' action. To better achieve the proposed research goals, we first performed in vitro studies to

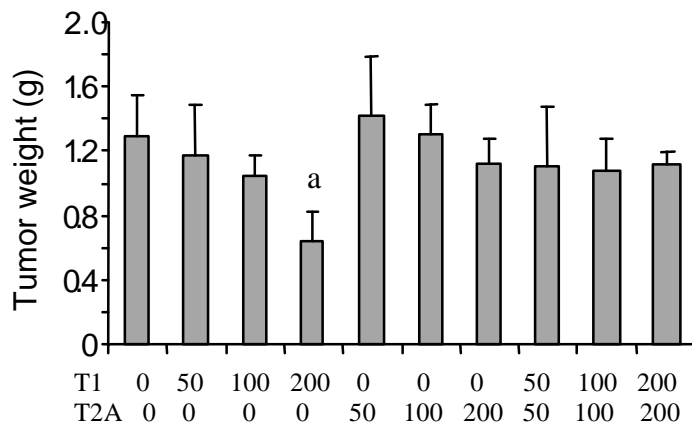


Figure 5. Effects of T1, T2A, and T1 and T2A combinations on the progression of androgen-independent LNCaP tumors after castration in the orthotopic LNCaP tumor animal model. The value with a superscript letter is significantly different from the control (a, $P < 0.05$).

identify candidate molecular biomarkers associated with the tanshinone treatment. We then conducted gene function assays to identify the functional role of candidate molecular target(s) in explaining the tanshinone activity. We further determined the cellular biomarkers and identified molecular target(s) in the in vivo samples to verify in vivo mechanisms.

To investigate the cellular and molecular mechanisms by which tanshinones, especially T1, inhibit the growth of prostate cancer, we conducted the following experiments: (1) we first determined the cellular mechanisms by which tanshinones inhibit the growth of prostate cancer cells in vitro; (2) we further determined molecular markers associated with cellular mechanisms;

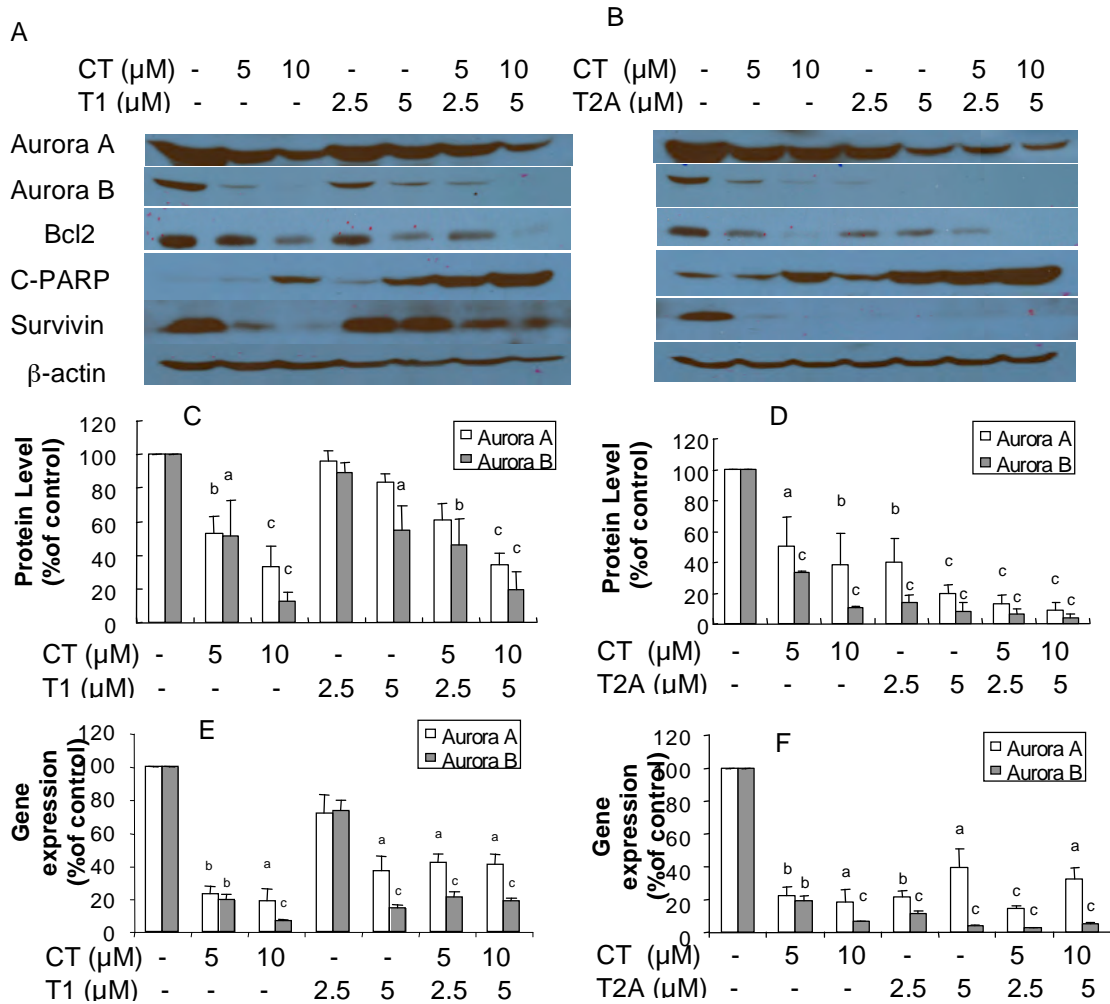


Figure 6. Effects of CT/T1 combinations (A) and CT/T2A combinations (B) on protein levels of Aurora A, Aurora B and apoptosis related biomarkers (bcl2, C-PARP, and survivin) in LNCaP cells. The corresponding imaging quantitation results of Aurora A and Aurora B protein levels are shown in C and D, respectively. The effects of CT/T1 combinations (E) and CT/T2A combinations (F) on the expression of Aurora A and Aurora B genes are also shown. In each panel, values with superscript letters are significantly different from the control (a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.001$).

(3) by applying real time PCR-based signaling pathway-specific array assays, we identified potentially novel molecular markers; (4) we further applied gene function-based assays to determine the functional role of identified molecular target in anti-prostate cancer activity of tanshinones. The major findings are summarized as follows:

1) Induction of prostate cancer cell apoptosis was an important cellular mechanism and

T1 showed the most potent apoptosis-inducing activity among three tanshinones (Figure 2a in appendix 1). In addition, the T1/CT or T2A/CT combination synergistically inhibited the growth of prostate (LNCaP) cancer cells (Fig 4A-D) in part via induction of LNCaP cell apoptosis in a synergistic manner (Figure 4 E-F).

2) Apoptosis-inducing activity of T1 was associated with modulation of several apoptosis-related molecular markers, such as Bcl2 and bax (Fig 2 of Appendix 1).

3) The real time PCR-based array assays identified Aurora A and Aurora B as two molecular biomarkers that were significantly modulated (downregulated) by tanshinones. Furthermore, the CT/T1 or CT/T2A synergistic combination also downregulated gene expression and protein levels of Auroras A and B in a synergistic manner (Figure 6).

4) Further studies confirmed that Aurora A gene expression and protein level were downregulated in prostate cancer cell lines by tanshinones (Figure 3A-3D, Appendix 1) in vitro and in DU145 prostate tumors in vivo (Figure 6 of Appendix 1).

5) Gene function assays showed that knockdown of Aurora A gene by siRNA significantly reduced the PC-3 (Fig 3E, F, Appendix 1) and LNCaP (Figure 7A) prostate cancer cell growth. Aurora A silencing also reduced the activity of CT or T1 in inhibiting the growth of

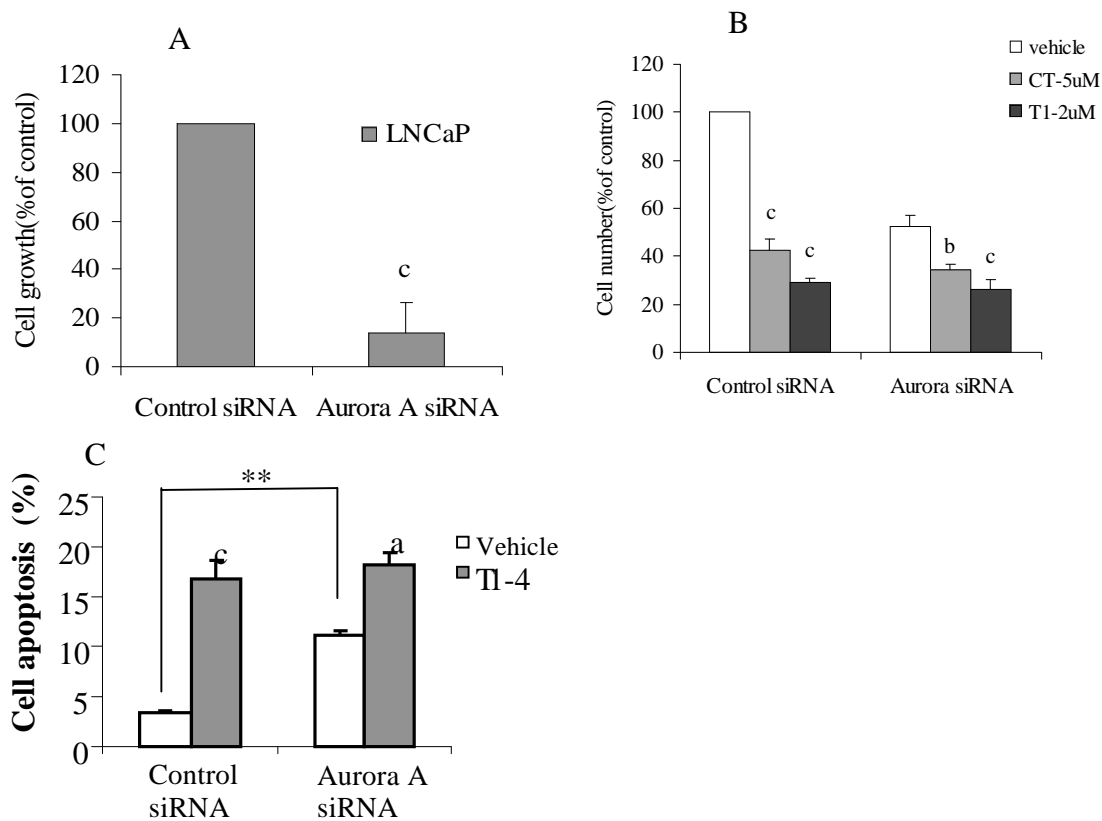


Figure 7. Effects of Aurora A siRNA on LNCaP cell growth inhibition (A) and on the activity of CT and T1 (B). Aurora A siRNA downregulated Aurora A gene expression and could inhibit LNCaP cell growth by 85% (A) at a higher dose. At a lower dose at which cell growth was inhibited by about 50% (for sensitive evaluation of siRNA effect on tanshinone activity), Aurora A silencing reduced the activity of CT (from 60% inhibition to 25% inhibition at 5 μ M) and T1 (from 70% to 50% at 2 μ M) (B). Aurora A silencing also caused increased apoptosis of prostate cancer cells and alleviated apoptosis-induction activity of T1 (C). Within panels, values with a letter are significantly different from the respective controls (a, P<0.05; b, P<0.01; c, P<0.001; ***, P<0.001).

LNCaP cells in vitro (Figure 7B).

6) Further assays showed that Aurora A silencing also caused an increased level of apoptosis of prostate cancer cells, and alleviated the apoptosis-inducing activity of tanshinones (T1) (Figure 7C).

7) In summary, our mechanism studies indicated that induction of apoptosis is an important cellular mechanism by which tanshinones inhibit the growth of prostate cancer cells in vitro and in vivo, and downregulation of Aurora A (and also possibly Aurora B, experiments not done) may be an important molecular mechanism by which tanshinones inhibited the growth of prostate cancer cells via induction of cancer cell apoptosis. Our preliminary studies also suggest that downregulating Aurora A (and/or B) function may also be an important molecular mechanism by which the T1/CT or T2A/CT combination synergistically inhibits prostate cancer cell growth.

3.2. Addition studies to identify functional molecular target(s) of tanshinones (directly related to specific aim 3)

In addition to our effort to define Aurora A as an important functional molecular target of tanshinones, we also initiated experiments to determine if another molecular biomarker, SIRT1, was a potential functional target of tanshinones. Our array assay results identified SIRT1 as another top candidate molecular biomarker. Therefore, we further performed a series of studies to determine the functional significance of Sirt1 in tanshinone actions in inhibiting prostate cancer growth. The results are summarized as follows.

1) Prostate cancer cells had increased SIRT1 expression and tanshinones (especially CT) upregulated SIRT1 expression. To determine the SIRT1 function in prostate cancer, we first measured gene expression and protein level of SIRT1 in prostate cancer cells and PrEC. Compared with the PrEC, human prostate cancer cells had overexpressed SIRT1 gene (Fig. 8A) and increased protein levels (Fig 8B). We further determined the effects of tanshinones on regulation of SIRT1 expression and found that tanshinones, especially CT significantly upregulated SIRT1 gene expression in prostate cancer cells (Fig. 8C).

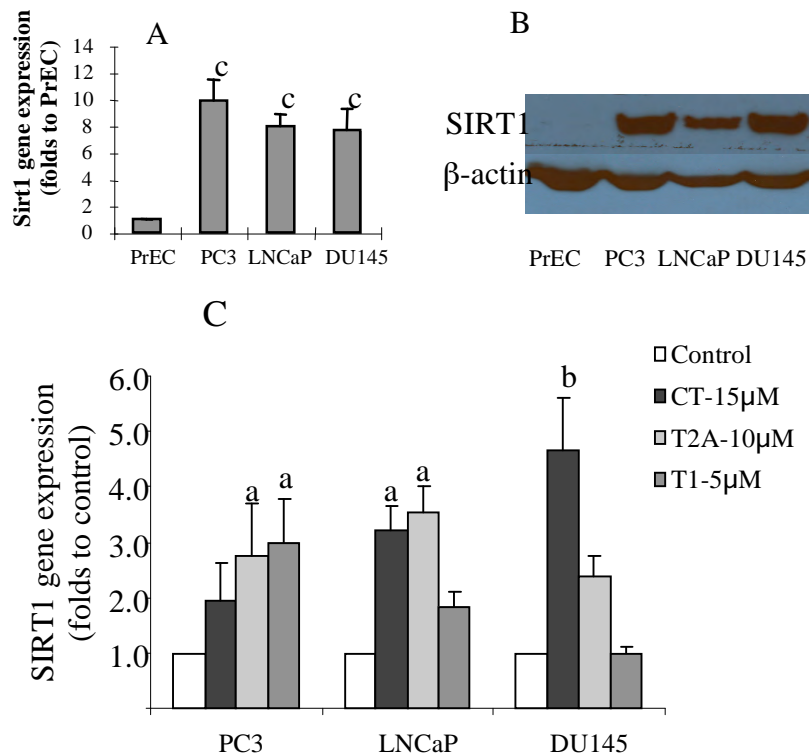
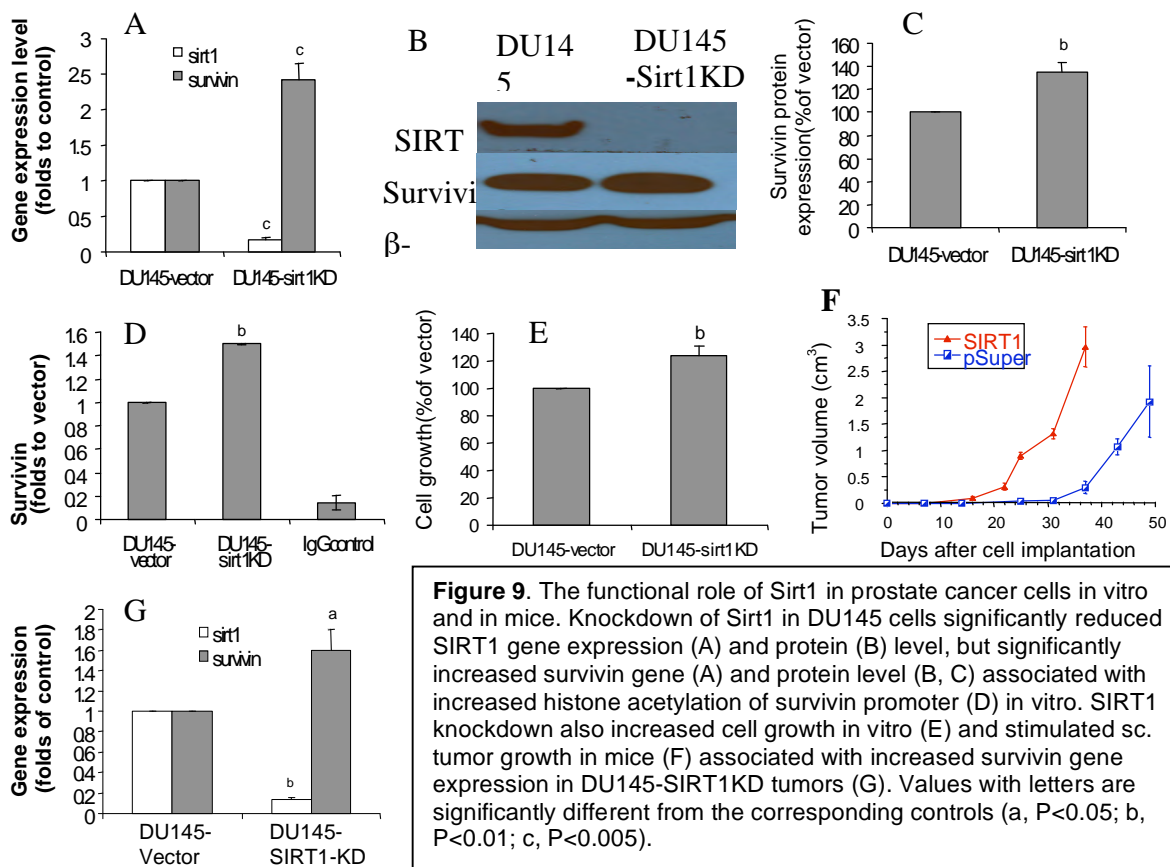
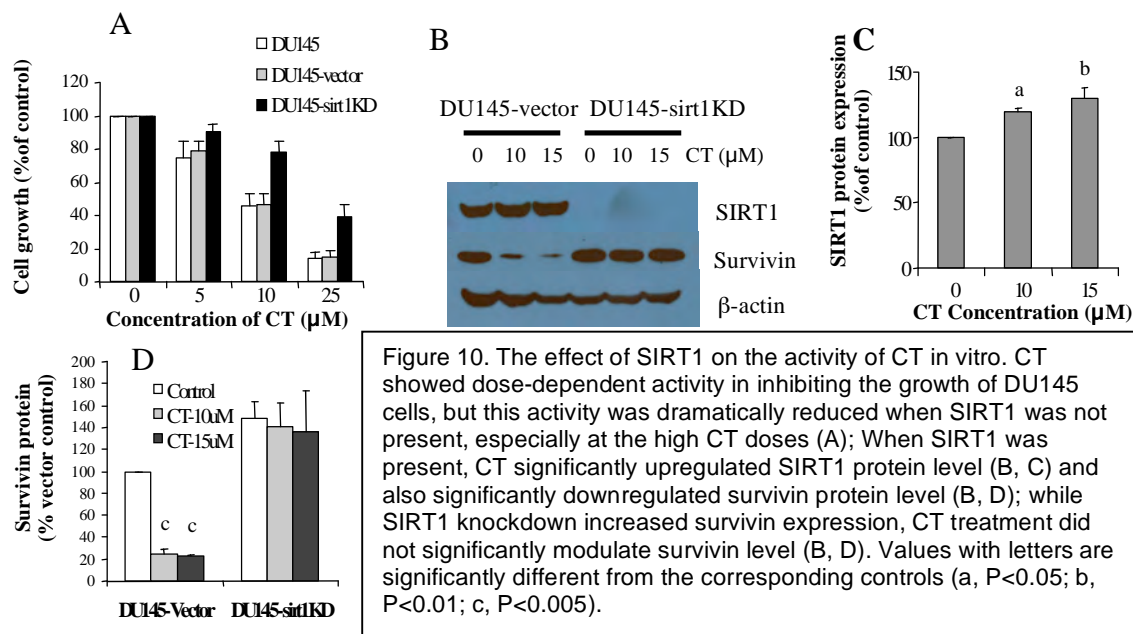


Figure 8. Overexpression of Sirt1 (A) and protein levels (B) in human prostate cancer cell lines, compared with that in PrEC. Tanshinones (especially CT) upregulated Sirt1 gene expression in prostate cancer cells (C) in vitro. Within the panel, values with letters are significantly different from the corresponding controls (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$).

2) SIRT1 knockdown stimulated the growth of prostate cancer cells in vitro and in vivo. Although SIRT1 was overexpressed in prostate cancer cells, it DOES NOT necessarily mean that SIRT1 plays an oncogenic role in prostate cancer cells, and the functional role of SIRT1 needs to be determined by functional assays. To determine the functional role of SIRT1, we established the SIRT1 knockdown stable cell line of DU145 (DU145-SIRT1-KD) and the vector control (DU145-pSuper). SIRT1 knockdown effectively reduced the SIRT1 gene expression (Fig 9A) and SIRT1 protein level (Fig 9B), and simultaneously increased survivin, a downstream target of Sirt1, gene expression by 150% (Fig 9A) and survivin protein level by 40% (Fig. 9B, 9C), suggesting that survivin is a direct downstream target of SIRT1. We used chromatin immunoprecipitation to measure the histone acetylation level in survivin gene promoter in both DU145-vector and DU145-SIRT1-KD cell lines, and found that SIRT1 knockdown significantly increased histone H3 acetylation level in survivin promoter, compared with the DU145-vector (Fig.9D, $P < 0.01$), a finding consistent with the histone deacetylase activity of SIRT1. It was further shown that SIRT1 knockdown increased the growth rate of DU145 cells in vitro by 30% compared with the vector control (Fig. 9E, $P < 0.01$), and more dramatically stimulated the growth of subcutaneous DU145 tumors in mice (Fig. 9F). The DU145-SIRT1-KD cells developed tumor over twice faster than the DU145-pSuper cells (14 days vs. 30 days for the first tumor to appear) and DU145-SIRT1-KD tumors also grew much faster than the vector controls (Fig 9F). As confirmed, the DU145-SIRT1-KD tumors had reduced SIRT1 gene expression by over 80% (Fig 9G) and increased survivin gene expression by 60% (Fig. 9G), compared with the vector control tumors. These results strongly suggest the tumor suppressive role of SIRT1 in prostate cancer growth and progression in part by downregulating survivin expression.



3). SIRT1 was a functional target of CT in inhibiting the growth of prostate cancer cells in vitro and in vivo. After establishing the functional (tumor suppressive) role in prostate cancer progression, we further determined if the growth inhibitory effect of CT on prostate cancer cells was in part via upregulation of SIRT1 expression. We first determined if SIRT1 knockdown would influence (reduce) the CT activity in vitro, and found that SIRT1 knockdown significantly reduced the activity of CT in inhibiting the growth of DU145 cells (Fig 10A). We also showed that, in the DU145-pSuper cells, CT upregulated SIRT1 expression (Fig 10B, 10C) and also dramatically downregulated survivin protein level (Fig 10B, 10D), whereas in the DU145-SIRT1-KD cells CT did not significantly downregulate survivin protein level (Fig 10B, 10D). These results demonstrated that when SIRT1 was present, it served as an important molecular target for CT action in part via subsequent downregulation of survivin function, whereas when SIRT1 was absent, CT still had certain activity via regulating other target(s).



KEY RESEARCH ACCOMPLISHMENTS

The following results were obtained from this research project:

- (1) The potent anti-prostate cancer cell growth activities of tanshinones were defined in the in vitro studies; among tanshinones, T1 showed the most potent activity.
- (2) The CT and T1 combination or the CT and T2A combination, but not the T1 and T2A combination, showed synergistic effect on inhibiting the growth of prostate cancer cells in vitro.
- (3) Tanshinones, especially T1, also showed potent anti-angiogenesis activity in the in vivo model, and the anti-angiogenic activity was further confirmed in the animal studies.
- (4) The rationale of the proposed synergistic effect of T1 and T2A combination was based on the observation that T1 and T23A showed the potent activities in inhibiting angiogenesis and prostate cancer cell growth, respectively. However, the in vivo animal studies did not find the synergistic or additive effects of T1 and T2A

- combination regimens against prostate tumor growth. This unexpected result may be in part due to the fact that the T1 and T2A combination may have antagonistic effect on inhibiting the growth of prostate cancer cells, as identified in vitro.
- (5) Importantly, the in vivo animal studies identified the significant effects of T1 and CT on inhibiting the growth of prostate tumors. Considering the observation that the CT and T1 combination had synergistic effect on inhibiting prostate cancer cell growth in vitro, the T1 and CT combination may also have synergistic effect, and this combination regimen should be further evaluated in the in vivo models.
 - (6) Despite its potent activity in vitro, T2A did not show significant activity in inhibiting the growth of LNCaP tumors in vivo. It is unclear at this time what caused this insignificant activity, and further investigation is required.
 - (7) Tanshinones showed minimal side effects on mice and did not significantly alter food intake or body weight. In fact, tanshinones, especially T1 slightly increased food intake of mice.
 - (8) Tanshinones inhibited the growth of prostate cancer cells in part by inducing apoptosis associated with modulation of apoptosis related biomarkers (such as bcl2, bax, survivin and Auroras A and B).
 - (9) Further gene function assays for Aurora A (Aurora B function was not performed) showed that knockdown of Aurora A by siRNA reduced prostate cancer cells growth and increased prostate cancer cell apoptosis, and alleviated the activity of tanshinones (T1) in inducing apoptosis and inhibiting the proliferation of prostate cancer cells. These results suggest Aurora A as an important functional molecular target for tanshinones actions.
 - (10) In addition to Aurora A, both in vitro and in vivo gene function assays also identified SIRT1 as another important molecular target for tanshinones actions.

REPORTABLE OUTCOMES

The following manuscript has been published:

Y. Gong, Y. Li, Y. Lu, L. Li, H. Abdolmaleky, G.L. Blackburn, J.-R. Zhou: Bioactive tanshinones in *Salvia Miltiorrhiza* inhibit the growth of prostate cancer cells in vitro and in mice. *Int. J. Cancer* 129:1042-52, 2011.

The following manuscripts are submitted for publication consideration:

- (1) Y. Gong, H. Abdolmaleky, L. Li, G.L. Blackburn, Y. Dai and J.-R. Zhou: Bioactive cryptotanshinone inhibits the growth of prostate cancer cells in part by upregulation of SIRT1 function in vitro and in mice. Submitted to *Cancer Res*
- (2) Y. Gong, L. Li, H.M. Abdolmaleky, G.L. Blackburn, J.R. Zhou: Tanshinones inhibit androgen-dependent prostate cancer via downregulation of aurora A and suppression of androgen-receptor signaling. Submitted to *Cancer Prevention Research*
- (3) R.-W. Jiang, H.-Y. Tian, T.-F. Chen, W. Ye, L. Li, F. Ni, J.-R. Zhou: Construction and screening of fractional library of *Salvia Miltiorrhiza* for the rapid identification of active cancer-preventive compounds. Submitted to *ChemBioChem*

The following manuscripts are being prepared and will be submitted for publication consideration:

(1) Synergistic tanshinone combinations for prostate cancer therapy

The results have been presented at the following scientific meeting:

- (1) J.R. Zhou: Synergistic combinations of active components in Danshen for prevention and treatment of prostate cancer. World Cancer Congress 2008, Shanghai, China.
- (2) J.R. Zhou: Combination of Active Components in Danshen for Prostate Cancer Treatment by Acting on Multiple Targets. Bio-Pearl River Forum and the 14th Annual Conference of Chinese Biopharmaceutical Association, Guangzhou, China, 2009
- (3) Gong Y, Li Y, Zhou J-R. Bioactive tanshinones in Salvia Miltiorrhiza for prostate cancer chemoprevention. Frontiers in Cancer Research 2009 (abstract #394); Houston, TX, December 6-9.
- (4) Zhou J-R, Li Y, Gong Y, Blackburn GL. Identification of bioactive dietary and natural components for targeting cancer stem cells. Experimental Biology annual meeting, Anaheim, CA, April 24-28, 2010.
- (5) Jin-Rong Zhou, Yi Gong, Linglin Li, Yanli Li, Hamid Abdolmaleky. Bioactive tanshinones as effective therapeutic and preventive agents for prostate cancer progression. 2011 Innovative Minds in Prostate Cancer Today (IMPACT) Conference, March 9-12, 2011, Orlando, FL

CONCLUSIONS

Our experiments defined T1 and CT as potent anti-prostate cancer growth candidate agents with minimal side effect. Although we did not verify the proposed synergistic effect of the T1/T2A combination regimen, our studies provided experimental evidence to suggest that the CT and T1 combination may have synergistic effect and this combination regimen should deserve further investigation.

REFERENCES

None

APPENDICES

Appendix 1: International J. Cancer 129:1042-52, 2011

Bioactive tanshinones in *Salvia Miltiorrhiza* inhibit the growth of prostate cancer cells *in vitro* and in mice

Yi Gong, Yanli Li, Yin Lu, Linglin Li, Hamid Abdolmaleky, George L. Blackburn and Jin-Rong Zhou

Nutrition/Metabolism Laboratory, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

Searching for efficacious and safe agents for the chemoprevention and therapy of prostate cancer has become the top priority of research. The objective of this study was to determine the effects of a group of tanshinones from a Chinese herb *Salvia Miltiorrhiza*, cryptotanshinone (CT), tanshinone IIA (T2A) and tanshinone I (T1) on prostate cancer. The *in vitro* studies showed that these tanshinones inhibited the growth of human prostate cancer cell lines in a dose-dependent manner via cell cycle arrest and apoptosis induction. Among three compounds, T1 had the most potent activity with IC₅₀s around 3–6 μM. On the other hand, tanshinones had much less adverse effects on the growth of normal prostate epithelial cells. The epigenetic pathway focused array assay identified Aurora A kinase as a possible target of tanshinone actions. The expression of Aurora A was overexpressed in prostate cancer cell lines. Moreover, knockdown of Aurora A in prostate cancer cells significantly decreased cell growth. Tanshinones significantly downregulated the Aurora A expression, suggesting Aurora A may be a functional target of tanshinones. Tanshinones, especially T1, also showed potent anti-angiogenesis activity *in vitro* and *in vivo*. Furthermore, T1 inhibited the growth of DU145 prostate tumor in mice associated with induction of apoptosis, decrease of proliferation, inhibition of angiogenesis and downregulation of Aurora A, whereas it did not alter food intake or body weight. Our results support that T1 may be an efficacious and safe chemopreventive or therapeutic agent against prostate cancer progression.

Prostate cancer is the most common invasive malignancy and the second leading cause of cancer death in America men. Current therapeutic modalities for prostate cancer usually have variable effectiveness and develop metastasis and drug-resistance associated with high toxicity to normal tissues. Therefore, the searching for more effective regimens with minimal adverse effects for the chemopreventive intervention of prostate cancer remains the top priority in prostate cancer research.

Key words: prostate cancer, tanshinones, Aurora A, angiogenesis, apoptosis, proliferation, chemoprevention

Abbreviations: cdc2: cell division cycle-2; CDK1: cyclin dependent kinase 1; CT: Cryptotanshinone; MVD: microvessel density; PrEC: Prostate epithelial cells; T2A: Tanshinone IIA; T1: Tanshinone I; TUNEL: terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling

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Yin Lu's present address is: Department of Pharmacy, Nanjing University of Traditional Chinese Medicine, Nanjing, Jiangsu, People's Republic of China

Correspondence to: Jin-Rong Zhou, PhD, Nutrition/Metabolism Laboratory, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, DA-881, 330 Brookline Avenue, Boston, MA 02215, USA, Tel.: (617)667-1280, Fax: (617) 667-1288, E-mail: jrzhou@bidmc.harvard.edu

The use of plants for medicinal purposes is as old as human history. All traditional and indigenous healing systems used natural products to treat or prevent disease. The use of medicinal botanical products is growing in the United States and many other countries. Most, if not all, traditional medical systems rely primarily on botanicals as a mainstay of therapy or prevention. Plants and other botanicals have also been the basis for most modern pharmaceutical drugs.

Herbal medicines usually contain multiple bioactive components with specific biological activities and are also used as alternative therapeutic or preventive regimens for individuals with cancer.¹ Some of those herbal medicines have been used for centuries without demonstrating significant adverse effects on humans, thus their active ingredients could serve as efficacious and safe candidates for the prevention and/or therapy of cancer.

Danshen (*Salvia miltiorrhiza* Bunge) has been widely used in traditional Chinese medicine practice for over a 1,000 years in the treatment of coronary artery disease and cerebrovascular diseases with minimal side effects.² Cryptotanshinone (CT), Tanshinone IIA (T2A) and Tanshinone I (T1) are three major diterpene compounds of tanshinones in Danshen.² In addition to their functions in cardiovascular systems, tanshinones have been recently shown to possess some activities against human cancer cells. T1 inhibited the growth of leukemia,^{3–6} lung⁷ and breast cancer⁸ *in vitro* in part via induction of apoptosis. T2A inhibited the growth of breast cancer,^{9,10} nasopharyngeal carcinoma,¹¹ glioma,¹² leukemia¹³ and hepatocellular carcinoma^{14,15} cells *in vitro* by induction of apoptosis,^{11,14} and inhibited the growth of hepatic carcinoma¹⁶ and breast tumor⁹ *in vivo*. T2A also

inhibited invasion of lung cancer cells *in vitro*.¹⁷ CT inhibited the growth of hepatocarcinoma cells¹⁸ *in vitro* via cell cycle arrest at S phase and the growth of gastric and hepatocellular cancer cells. However, there is no report about the effect of tanshinones on prostate cancer.

The objectives of this study were to systematically evaluate tanshinones as potential chemopreventive and therapeutic candidates against prostate cancer progression by using both *in vitro* and *in vivo* systems. Our results provided convincing experimental evidence to support the future development of tanshinones, especially T1, as efficacious and safe agents for the prevention and/or therapy of prostate cancer.

Material and Methods

Materials

Tanshinones CT, T2A and T1 were purchased from LKT Laboratories (St. Paul, MN), and the purities were verified by high performance liquid chromatography. Tissue culture media, fetal bovine serum (FBS), and trypsin were from Life Technologies, Inc. (Grand Island, NY). Propidium iodide (PI) was from Sigma (St. Louis, MO); RNase A and 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were from Promega (Madison, WI). Antibodies used in Western blot against human antigens were cyclin B1, cdc2 and Bax (Oncogene Research Products, Boston, MA), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), Aurora A (Cell Signaling, Beverly, CA) and β -actin (Merck Co., Darmstadt, Germany). Antibodies used for immunohistochemistry against human antigens were Ki67 and Factor VIII (Dako North America, Carpinteria, CA) and Aurora A (Abcam, Cambridge, MA). Biotinylated anti-mouse/anti-rabbit IgG, Vectastain ABC kit and DAB substrate kit were from Vector Laboratories (Burlingame, CA).

Cell culture

Androgen-sensitive LNCaP and androgen-independent PC-3 and DU145 human prostate cancer cells from the American Type Culture Collection (ATCC, Bethesda, MD) were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS and antibiotics in a humidified atmosphere of 95% air and 5% CO₂. Human normal prostate epithelial cells (PrEC) were purchased from Lonza (Walkersville, MD) and cultured in PrEGM plus EGM-2 singlequotes (Lonza, Walkersville, MD) in a humidified atmosphere of 95% air and 5% CO₂. Human umbilical vein epithelial cells (HUVEC) were purchased from Lonza (Walkersville, MD) and cultured in endothelia cell basal medium (EGM-2) plus EGM-2 singlequotes (Lonza, Walkersville, MD) in a humidified atmosphere of 95% air and 5% CO₂.

Cell growth assay

The effects of tanshinones on cell growth were determined by using Cell Titer 96 Aqueous One Solution Reagent, MTS

(Promega) as we previously used.¹⁹ The experiments were repeated at least twice, each in triplicate.

Clonogenic survival assay

The effects of tanshinones on clonogenic survival of cancer cells were determined by a colony-forming assay following the method we described before.¹⁹ The experiments were done at least twice, each in duplicate.

Cell cycle analysis

Cells treated with different concentration of tanshinones were harvested, stained with PI and then analyzed by flow cytometry (Becton Dickinson, Immunocytometry Systems, Mountview, CA) for cell cycle distribution according to the protocol we used before.^{20,21}

Cell apoptosis detection

Cell apoptosis after treatment with tanshinones was determined by Annexin V-PI apoptosis detection kit. In brief, treated cells (1×10^5) were collected by trypsinization and centrifugation. Cells were washed with phosphate-buffered saline (PBS) and resuspended in 500 μ L Annexin V binding buffer, to which 2.5 μ L Annexin V was added and incubated at room temperature for 15 min; then 10- μ L PI was added for another 5-min incubation in the dark. Apoptotic cells were analyzed by flow cytometry (Becton Dickinson, Immunocytometry Systems, Mountview, CA).

Cancer cell invasion assay

The effect of tanshinones on prostate cancer cell invasion was determined using a BD biocoat matrigel invasion chamber. PC-3 cells were starved overnight in serum-free medium and 200 μ L of cell suspension (2.5×10^5 cells/mL) with or without tanshinones were added to the upper chamber. The lower chamber was filled up with 750 μ L of NIH3T3 fibroblast conditioned media. The cells were incubated for 20 hrs at 37 °C. After incubation, cells in the top well were removed carefully by swiping the cotton swabs and the cells on the underside of the membrane were stained using the Diff Quik (Dade Behring) stain kit, and quantified. The treatment was duplicated and the experiment was repeated at least twice.

Western blot analysis

Cells were treated with different concentrations of tanshinones, cell lysates were prepared, and protein expression was determined following the procedures we previously described.^{19,21} The primary antibodies used were as follows: cyclin B1 (1:200), Cdc-2 (1:500), Bcl-2 (1:100), Bax (1:500), Aurora A (1:1000) and β -actin (1:10,000).

Epigenetic chromatin modification enzymes PCR array

The Human Epigenetic Chromatin Modification Enzymes RT² ProfilerTM PCR Array (SABiosciences, Frederick, MD) was used to detect the expression of 84 key genes encoding enzymes known or predicted to modify genomic DNA and

histone to regulate chromatin accessibility and therefore gene expression. The experiment was performed according to the vendor's instruction.

Quantitative real time reverse transcription-PCR

Total RNA was isolated by using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA synthesis used 100 ng random primer (Invitrogen, Carlsbad, CA), 1.0 µg of total RNA, 10 mM dNTP and 200 units of reverse transcriptase (Invitrogen, Carlsbad, CA) per 20 µL reaction. The sequences of primers used in this study are listed as follows:

β-actin forward 5'-GATGAGATTGGCATGGCTTT-3',
reverse 5'-CACCTTCACCGTTCCAGTTT-3' with a product size of 100bp;

Aurora A forward 5'-CATCTTCCAGGAGGACCACT-3',
reverse 5'-CAAAGAACTCCAAGGCTCCA-3' with a product size of 112bp. PCRs were performed in duplicates in a 25 µL final volume by using SYBR Green master mix from SABiosciences (Frederick), and the data were analyzed by using the same methods as described above.

Aurora A silencing by siRNA

The Aurora A silencing by siRNA followed the method described by Lentini *et al.*²² with appropriate modifications. Briefly, 8×10^4 PC-3 cells were seeded in a 6-well plate and incubated for 24 h. The silencer negative control and siRNA for Aurora A (Ambion, Austin, TX) were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA) and transfected with Lipofectamine 2000 according to the manufacturer's instructions. The final concentration of siRNA added to the cells was 33 nM. The duplex siRNA sequence for Aurora A was as follows: 5'-AUGCCCUGUCUUACUGUCATT-3'.

In vitro angiogenesis assays

The HUVEC proliferation, migration and tube formation assays were used as the *in vitro* angiogenesis assays. HUVEC proliferation followed the MTS assay as described before.¹⁹

For the endothelial cell migration assay, the BD fibronectin Biocoat 24-well chambers (3-µm pore size) were used. The lower chamber was loaded with 650 µL of complete medium (vascular endothelial growth factor, platelet-derived growth factor and insulin-like growth factor as chemoattractants). The upper chamber wells were loaded with HUVEC cells (50,000 cells/well) in a 200 µL of serum-free M199 medium with 0.1% bovine serum albumin. The cells were then treated with tanshinones or the vehicle and incubated for 5 h at 37 °C, and the migrated cells were measured following the method described in the cancer cell invasion assay.

For the tube formation assay, matrigel (BD Bioscience, City, CA) was added (50 µL) to each well of a 96-well plate and incubated at 37 °C for 1 h to solidify. A suspension of HUVEC cells (12,500 cells) in EGM2 medium were seeded into each well and treated with tanshinones or the vehicle.

After 18 h of incubation at 37 °C, images were captured, and tubes formed were scored as follows: a three-branch point event was scored as one tube.

In vivo matrigel plug assay

The *in vivo* matrigel plug assay followed the protocol described by Huh *et al.*²³ with appropriate modifications. Male C57BL/6J mice (8-week-old) were purchased from Taconic (Germantown, NY), randomized into the Control or the T1 treatment group ($n = 8$ /group) and fed the AIN-93M diet. After 2 weeks of treatment with either the T1 (50 mg/kg BW, in corn oil) or the vehicle (corn oil) via gavage, each mouse was injected subcutaneously with 0.5 mL of matrigel (BD Sciences) and continued the treatment for 7 days. The mice were sacrificed, and the matrigel plugs were excised and weighed. Hemoglobin levels in matrigel plugs were measured as an indication of blood vessel formation, using the Drabkin method (Sigma, St. Louis). The concentration of hemoglobin was calculated from a hemoglobin standard curve.

Animal study

Male SCID mice (8-week-old) were purchased from Taconic (Germantown, NY), and fed the AIN-93M diet for one week of adaptation. Mice were randomly assigned into two experimental groups (control and T1 treatment), and each mouse was inoculated subcutaneously with 2×10^6 of PC-3 cells and treated with the assigned experimental treatment, either the vehicle (100 µL corn oil) or T1 in corn oil at 150 mg/kg BW by gavage daily. Food intake and body weight were measured weekly. The tumor diameters were measured weekly.²¹ At the end of the experiments, the mice were sacrificed; primary tumors were excised and weighed. A tumor slice from each primary tumor tissue was carefully dissected and fixed in 10% buffer-neutralized formalin, paraffin-embedded and sectioned at 4-µm thickness for immunohistochemistry.

In situ detection of apoptotic index

Apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay (Chemicon International, Billerica, MA) following our described protocols.^{20,24} The staining was analyzed by using the Ivision imaging software (Biovision, Exton, PA).

Immunohistochemical determination of tumor cell proliferation

The proliferation index was evaluated by calculating the proportion of cells with Ki-67 staining, following the procedures in the laboratory.²⁴

Immunohistochemical determination of microvessel density

Microvessel density (MVD) was used as a marker for tumor angiogenesis and detected by immunohistochemical staining of Factor VIII following a method described previously.^{20,24}

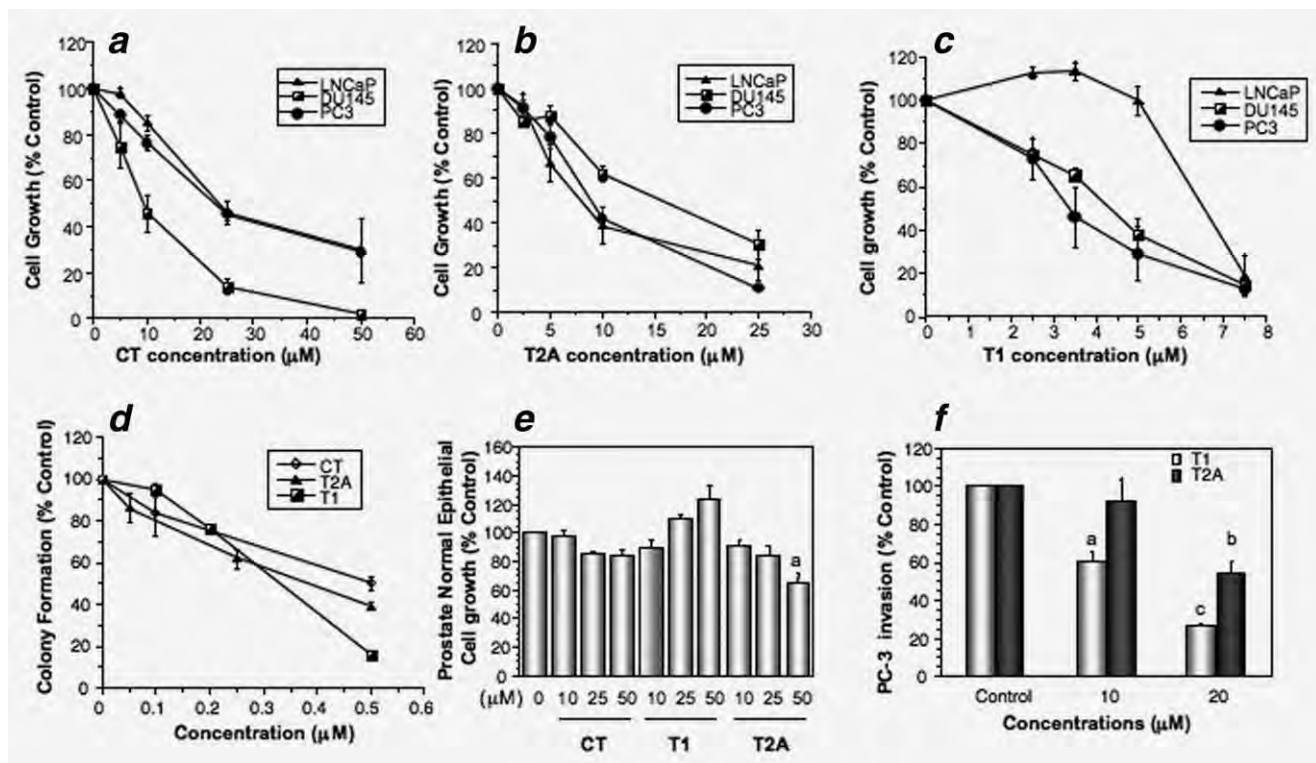


Figure 1. Effects of tanshinones on the growth, colony formation and invasion of human prostate cancer cells and the growth of normal PrEC *in vitro*. *a–c*, The dose-dependent effects of CT (*a*), T2A (*b*) and T1 (*c*) on the growth of androgen-sensitive LNCaP and androgen-independent DU145 and PC-3 cell lines. *d*, Effects of tanshinones on the growth of normal PrEC. *e*, Effects of tanshinones on colony formation of PC-3 cells. *f*, Effects of T2A and T1 on PC-3 cell invasion. Values are mean \pm SEM of three independent experiments in duplicate. Within the tanshinone treatment in each panel (*e* and *f*), the value with a letter is significantly different from that of the corresponding control, *a*, $p < 0.05$; *b*, $p < 0.01$; *c*, $p < 0.001$.

Representative images were captured and the data was analyzed by the Ivison imaging software (Biovision).

Immunohistochemical staining of Aurora A

The expression level of Aurora A in the tumor tissue was detected by immunohistochemistry following the protocols described previously^{20,24} with appropriate modifications. Briefly, after deparaffinization, rehydration and washing, the section was treated with 0.1% trypsin for 45 min for antigen retrieval. It was then incubated with diluted Aurora A antibody (1:200) overnight and then subjected to the secondary antibody incubation and staining procedures. Representative images were captured and analyzed by using the Ivison imaging software.

Statistical analysis

Results were expressed as group means \pm SEM and analyzed for statistical significance by analysis of variance followed by Fisher's protected least-significant difference based on two-side comparisons among experimental groups by using Statview 5.0 program (SAS Institute, Cary, NC). A $p < 0.05$ was considered statistically significant.

Results

Effects of tanshinones on the growth, clonogenic survival and invasion of prostate cancer cells *in vitro*

Tanshinones CT (Fig. 1*a*), T2A (Fig. 1*b*) and T1 (Fig. 1*c*) inhibited prostate cancer cell growth in a dose dependent manner. Among the three compounds, T1 showed the most potent activity with its IC_{50} 's around 3–6.5 μM , whereas the IC_{50} 's of CT and T2A are around 10–25 μM and 8–15 μM , respectively, for different prostate cancer cell lines (Fig. 1*a–c*).

We further examined the effects of CT, T2A and T1 on clonogenic survival of prostate cancer cells. Tanshinones significantly inhibited the colony formation of PC-3 (Fig. 1*d*) and other cell lines (LNCaP and DU145, data not shown). When compared to the growth inhibition assay, the colony formation was more sensitive (approximately 10 folds) to the treatment. On the other hand, tanshinones did not show significant cytotoxicity on normal PrEC at the concentrations high as 50 μM (Fig. 1*e*). The results suggest that tanshinones may have potent anti-growth effects on prostate cancer cells, but limited adverse effect on normal cells.

The effects of tanshinones on prostate cancer cell invasion were evaluated in highly invasive PC-3 cells. T2A and T1 inhibited PC-3 cell invasion in a dose-dependent manner, and T1 was more potent than T2A (Fig. 1*f*). At the current

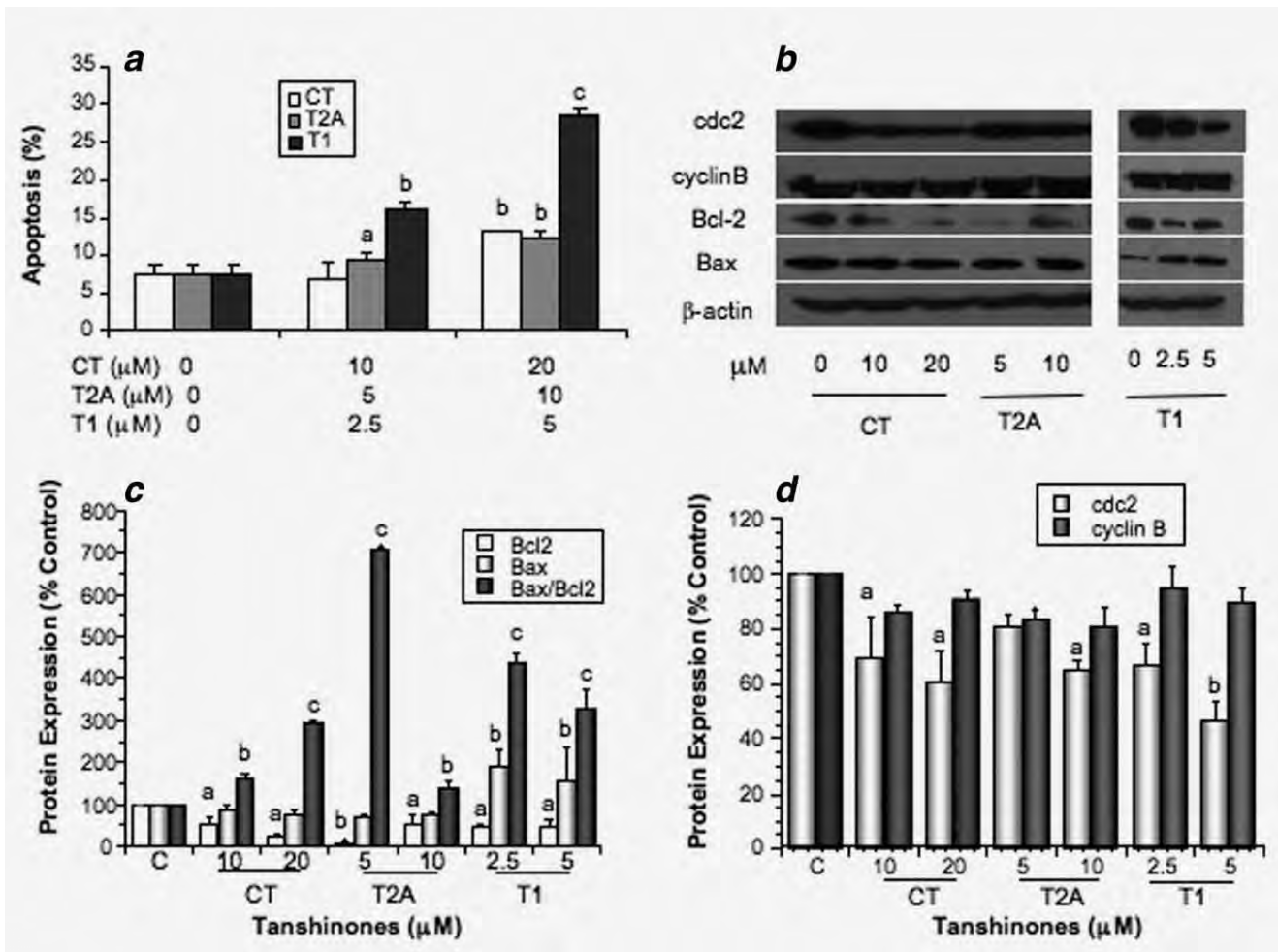


Figure 2. Effects of tanshinones on apoptosis of PC-3 cells measured by Annexin V-PI staining and flow cytometry (a), the expression of apoptosis related biomarkers bcl-2 and bax measured by Western blot (b) and quantified by densitometry after normalization to β -actin (c), and the expression of cell cycle related biomarkers cdc2 and cyclin B measured by Western blot (b) and quantified by densitometry after normalization to β -actin (d). Values are mean \pm SEM of three independent experiments in duplicate. Within the tanshinone treatment in each panel (a, c, or d), the value with a letter is significantly different from that of the corresponding control, a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

experimental conditions, T1 or T2A did not significantly inhibit the growth of PC-3 cells (data not shown).

Effects of tanshinones on PC-3 cell apoptosis *in vitro*

We used Annexin V-PI apoptosis detection kit to determine the effects of tanshinones on apoptosis induction of PC-3 cells. As shown in Fig. 2a, CT, T2A and T1 treatments induced apoptosis dose-dependently. Among three tanshinones, T1 was the most potent one in apoptosis induction and increased apoptosis by 6.5 folds at the concentration of 5 μ M.

To elucidate the molecular mechanisms of tanshinones activities in apoptosis induction, we measured the expression of Bax and Bcl-2 proteins (Fig. 2b, 2c). All tanshinones significantly downregulated the expression of Bcl-2 (p at least < 0.05) in PC-3 cells, however, only T1 significantly upregulated Bax expression (p at least < 0.05). All tanshinones significantly increased the Bax/Bcl2 ratio, a more reliable indicator of apoptosis (Fig. 2c, p at least < 0.05).

Effects of tanshinones on cell cycle progression *in vitro*

Cell cycle progression analysis showed that CT and T1 arrested cell cycle at S phase, whereas T2A arrested cell cycle at G2-M phases. Compared with the control PC-3 cells ($30.56 \pm 0.95\%$), cells treated with 10 and 20 μ M CT increased the S phase proportion to $34.46 \pm 1.07\%$ ($p > 0.05$) and $37.08 \pm 2.49\%$ ($p < 0.05$), respectively. Similarly, cells treated with 2.5 and 5 μ M T1 increased the S phase to $38.65 \pm 0.40\%$ ($p < 0.05$) and $39.87 \pm 1.37\%$ ($p < 0.05$), respectively. On the other hand, cells treated with 5 and 10 μ M T2A increased the G2-M phase distribution to $32.97 \pm 1.45\%$ ($p < 0.05$) and $37.94 \pm 1.93\%$ ($p < 0.05$) respectively, compared with the control cells ($28.68 \pm 3.66\%$).

We also measured the protein markers related to cell cycle progression, and the results showed that CT, T2A and T1 treatment significantly decreased the protein level of cdc2 ($p < 0.05$) in a dose dependent manner, but did not significantly alter the expression of cyclin B (Fig. 2d).

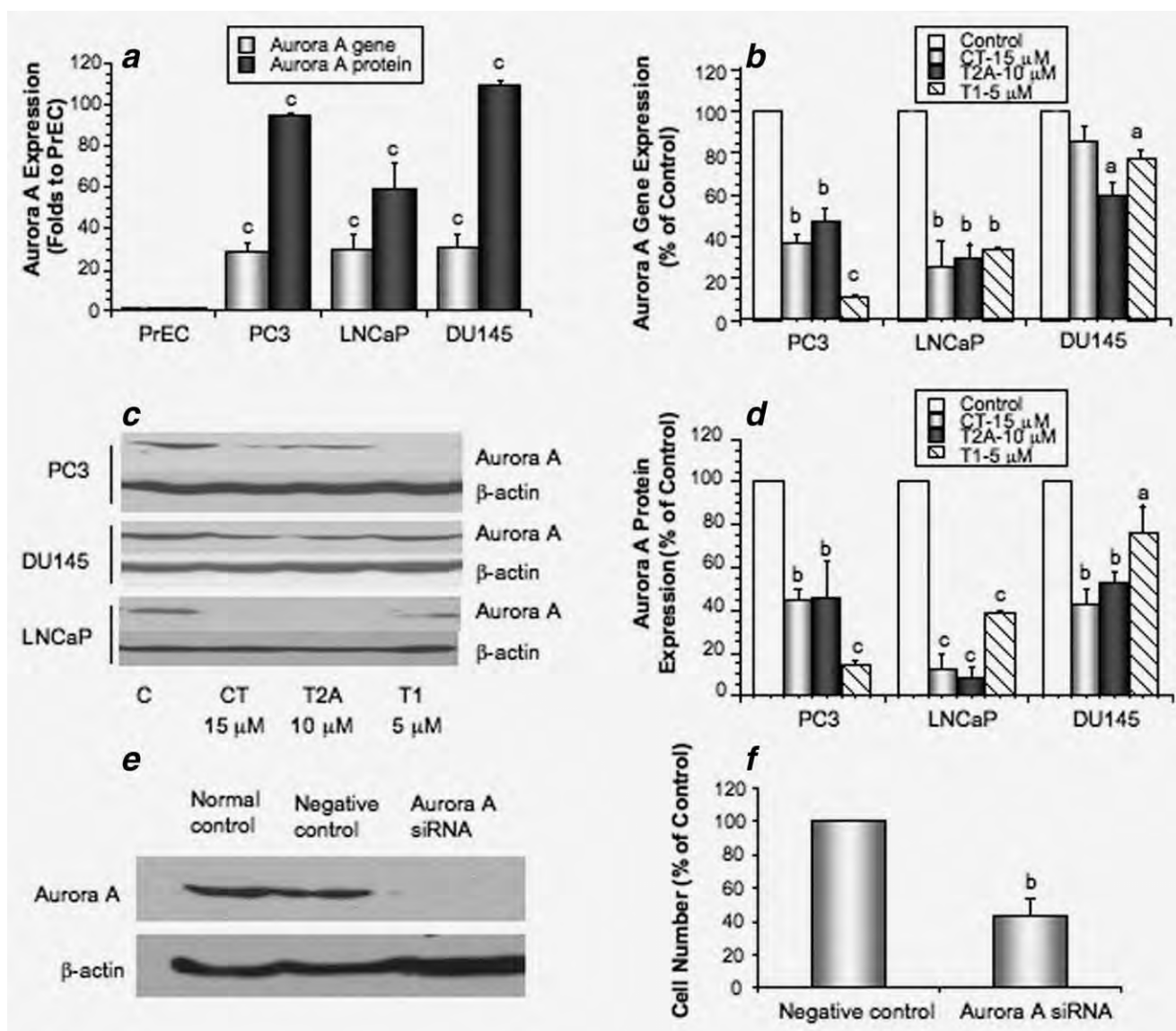


Figure 3. The gene and protein expression of Aurora A in prostate cancer cell lines and PrEC (a), the effects of tanshinones on the expression of Aurora A gene measured by real time PCR (b) and Aurora A proteins measured by Western blot (c) and quantified by densitometry (d), representative western blots showing the successful silencing of Aurora A by siRNA in PC-3 cells as confirmed by Western blot (e), and the effect of Aurora A knockdown on the growth of PC-3 cells (f). Values are mean \pm SEM of three independent experiments in duplicate. Within the panel, the value with a letter is significantly different from that of the corresponding control, a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

Effects of tanshinones on the expression of epigenetic modification related genes *in vitro*

To identify the target genes of CT, T2A and T1, PC-3 cells were treated with 15 μM CT, 7.5 μM T2A, or 5 μM T1, and then collected for the PCR array analysis. Only the genes with $\Delta\Delta\text{Ct}$ of 2 were considered as significant. Among the 84 genes related to epigenetic modification, 32 were downregulated by more than two folds after T1 treatment, including Aurora A kinase, DNA methyltransferase, Histone acetyltransferase, Histone deacetylase, Lysine (K)-specific demethylase, Protein arginine methyltransferase. However, CT or T2A treatment significantly downregulated only Aurora A kinase

gene. The results suggest that Aurora A may be a potential molecular target of tanshinone actions.

Effects of tanshinones on Aurora A expression *in vitro*

We compared Aurora A expression between normal PrEC and prostate cancer cell lines. Compared to PrEC, prostate cancer cell lines (PC-3, LNCaP, and DU145) had significantly overexpressed levels of Aurora A gene and protein (Fig. 3a, $p < 0.001$). Treatments of prostate cancer cell lines with tanshinones significantly downregulated the gene (Fig. 3b, p at least < 0.05) and protein (Fig. 3c, 3d, p at least < 0.05) levels of Aurora A.

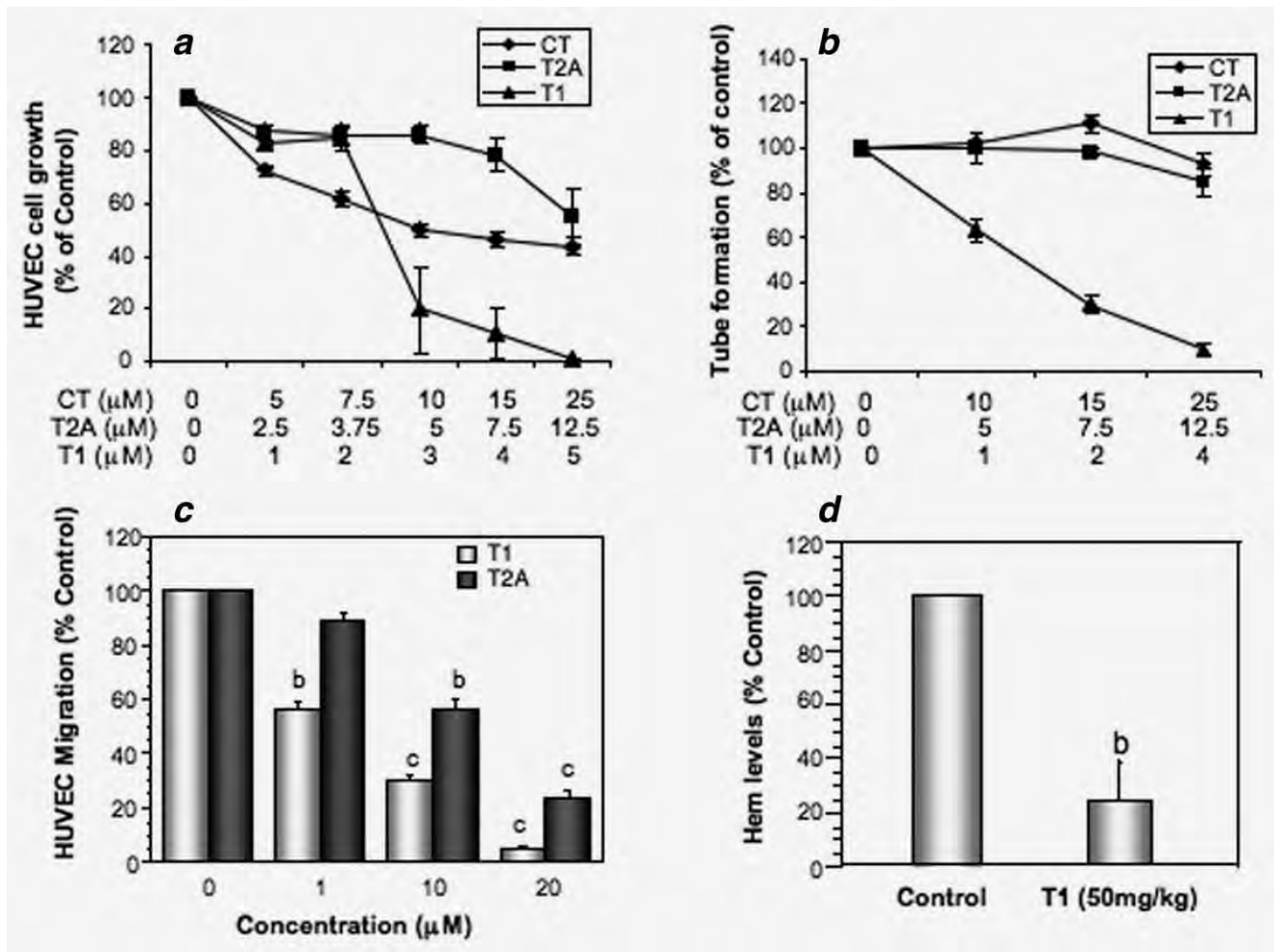


Figure 4. Effects of tanshinones on the growth (a), tube formation (b) and migration (c) of HUVEC *in vitro*, and the effect of T1 treatment at 50mg/kg BW on anti-angiogenesis *in vivo* measured by Matrigel Plug Assay (d). The *in vitro* values were mean \pm SEM of at least three independent experiments in duplicate. The *in vivo* values were the mean \pm SEM ($n = 8/\text{group}$). The value with a letter is significantly different from that of the corresponding control, a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

Aurora A function in prostate cancer cell growth *in vitro*

To determine the functional role of Aurora A in prostate cancer, we used Aurora A specific siRNA to downregulate its expression. Aurora A siRNA treatment effectively knocked down Aurora A protein level in PC-3 cells (Fig. 3e) by over 90%. Effective knockdown of Aurora A also significantly reduced the growth of PC-3 cells by over 50% (Fig. 3f, $p < 0.01$). The results suggest that Aurora A play an important function in prostate cancer cell growth.

Effects of tanshinones on angiogenesis *in vitro* and *in vivo*

The HUVEC cell growth, migration and tube formation were used as the *in vitro* angiogenesis assays. CT, T2A and T1 had potent activities in inhibiting the growth of HUVEC cells in a dose-dependent manner, with the IC_{50} s around 10 μM , 15 μM and 2.5 μM , respectively (Fig. 4a). CT and T2A had no apparent effect on tube formation, but T1 had a very potent activity in inhibiting tube formation with the IC_{50} less than 1

μM (Fig. 4b). Tanshinones (T1 and T2A) also inhibited HUVEC migration in a dose-dependent manner (Fig. 4c). Because T1 showed the most potent anti-angiogenesis activity *in vitro*, its anti-angiogenesis activity was further evaluated in the *in vivo* matrigel plug assay. T1 treatment (50mg/kg BW) significantly inhibited angiogenesis by 80% (Fig. 4d, $p < 0.01$). These results suggest that tanshinones, especially T1 may have potent anti-angiogenesis activity.

Effects of T1 treatment on DU145 tumor growth and modulation of tumor cell proliferation, apoptosis and Aurora A expression and tumor angiogenesis *in vivo*

Because T1 showed the most potent anti-prostate cancer cell growth *in vitro* and anti-angiogenesis activities *in vitro* and *in vivo*, we further evaluated the effect of T1 on prostate tumor growth in mice. As shown in Fig. 5a, T1 treatment (150 mg/kg BW) significantly inhibited the final tumor weight by 67% ($p < 0.05$). On the other hand, T1 did not significantly alter

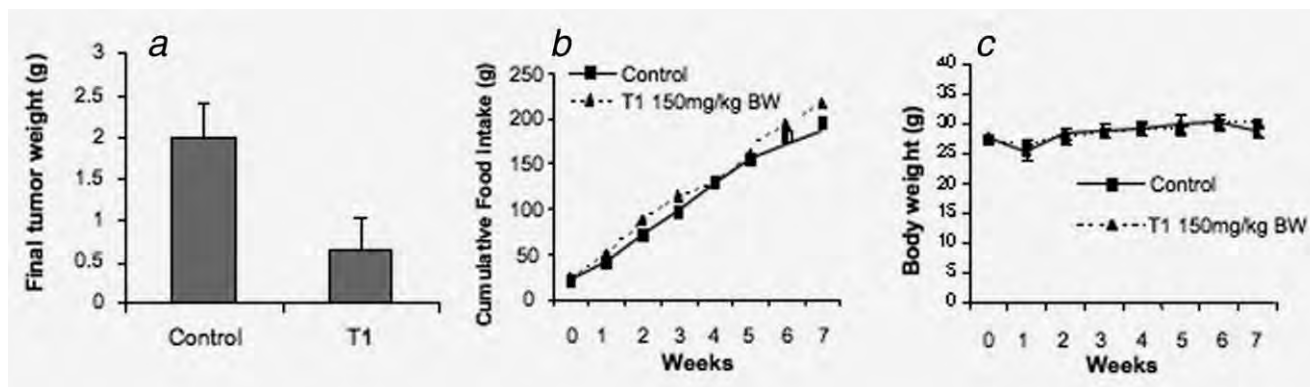


Figure 5. Effects of T1 treatment at 150mg/kg BW on the growth of DU145 tumors in mice (a), food intake (b) and body weight (c). Values are group mean \pm SEM ($n = 6$ /group).

either food intake (Fig. 5b) or body weight (Fig. 5c), suggesting that T1 treatment had limited adverse effect on mice.

Analyses of cellular markers showed that the T1 treatment significantly induced prostate cancer cell apoptosis by 250% (Fig. 6a, $p < 0.01$), reduced prostate cancer cell proliferation by 60% (Fig. 6b, $p < 0.01$) and inhibited prostate tumor angiogenesis by 80% (Fig. 6c, $p < 0.01$). The T1 treatment also significantly reduced Aurora A protein expression by 60% in prostate tumors (Fig. 6d, $p < 0.05$). These results confirmed that T1 inhibited the growth of prostate tumors by inducing apoptosis, reducing proliferation and downregulating Aurora A protein level of prostate cancer cells, and inhibiting prostate tumor angiogenesis *in vivo*.

Discussion

In this report, we conducted a series of *in vitro* studies and found that tanshinones CT, T1 and T2A significantly inhibited the growth of both androgen-sensitive and androgen-independent human prostate cancer cell lines in part via induction of apoptosis, cell cycle arrest at S phase (CT and T1) or G2-M phase (T2A) and downregulation of Aurora A expression. Knockdown of Aurora A by siRNA significantly reduced the growth rate of prostate cancer cells, suggesting that Aurora A may be a functional target for tanshinones. Tanshinones also inhibited angiogenesis in both the *in vitro* and *in vivo* assays. Among three tanshinones, T1 showed the most potent anti-growth, anti-invasion and anti-angiogenesis activities. The animal study further confirmed that T1 treatment significantly reduced the final tumor weight associated with induced prostate cancer cell apoptosis, reduced prostate cancer cell proliferation, downregulated prostate cancer cell Aurora A protein expression and inhibited prostate tumor angiogenesis, without altering food intake or body weight. This is the first report, to the best of our knowledge, that a novel group of bioactive tanshinones, especially T1, had potent anti-prostate cancer and anti-angiogenesis activities against prostate cancer progression. It is also the first report to identify Aurora A as the functional target for tanshinone actions.

Because prostate cancer has long latency and its risk increases with age, chemopreventive strategies could be applied to effectively prevent or delay its progression. However, the outcome from the recent selenium and vitamin E cancer prevention trial (SELECT trial) has been disappointing.²⁵ In the prostate cancer prevention trial (PCPT trial), finasteride, a 5 α -reductase inhibitor, reduced the risk of prostate cancer by 24.8%, but was initially associated with increased risk of high-grade disease by 25.5%.²⁶ Although reanalysis indicated that high-grade cancer was not associated with finasteride, the results may need further confirmation from another clinical trial, the Reduction by Dutasteride of prostate cancer events (REDUCE) trial.²⁷ Therefore, there is an urgency to identify more promising safe and efficacious agents for prostate cancer chemoprevention. Our results from the systematic *in vitro* and *in vivo* studies strongly suggest that tanshinone T1 may have favorable efficacy and safety profiles and may serve as a promising chemopreventive agent against prostate cancer progression.

Despite previous findings that tanshinones had anti-cancer activities, the molecular mechanisms remain elusive. Our studies support that downregulation of Aurora A may be an important molecular mechanism by which tanshinones possess chemopreventive activity against prostate cancer progression. Aurora kinases are a novel oncogenic family of mitotic serine/threonine kinases, which comprise three family members, Aurora-A, Aurora-B and Aurora-C.^{28,29} Aurora-A is localized on duplicated centrosomes and spindle poles during mitosis and is required for the timely entry into mitosis and proper formation of a bipolar mitotic spindle by regulating centrosome maturation, separation and microtubule nucleation activity.³⁰ Aurora A was frequently overexpressed in different types of cancers^{22,28,31-33} and in prostate cancer.^{31,34-37} Suppression of Aurora A expression and function reduced the prostate tumor growth and sensitized the activity of chemotherapeutic drugs.³⁸ Thus Aurora A has been recognized as an important molecular target for cancer therapy.^{39,40} Our studies demonstrated that tanshinones significantly down-regulated the gene and protein levels of Aurora A, supporting that Aurora A may be a novel molecular target for tanshinone actions.

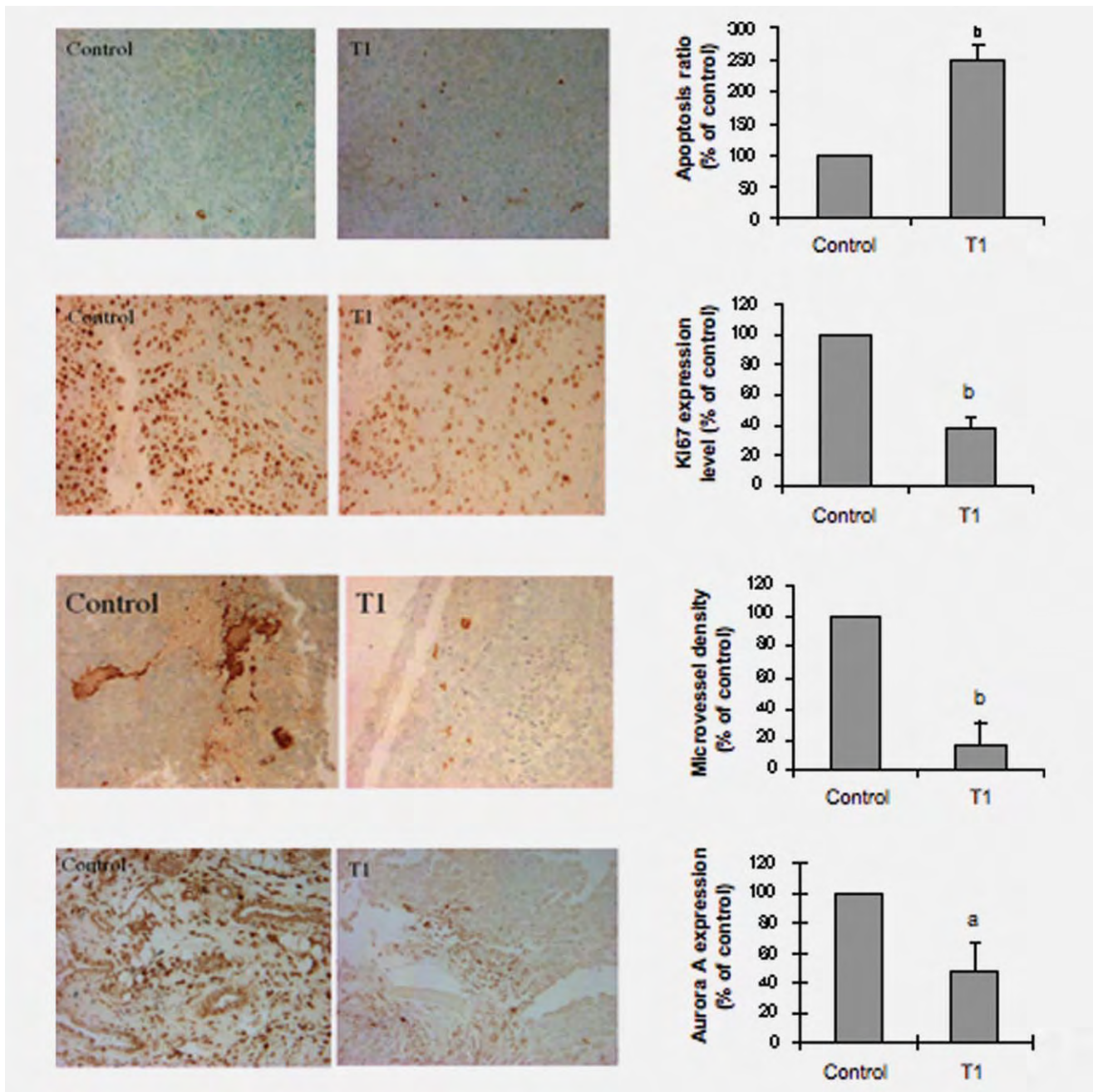


Figure 6. Effects of T1 treatment on DU145 tumor cell apoptosis index measured by TUNEL assay (a), tumor cell proliferation measured by immunohistochemical staining of ki-67 (b), tumor MVD measured by Factor VIII staining (c) and tumor cell expression of Aurora A measured by immunohistochemistry (d). For the analysis of immuno-staining, at least three representative areas of each section were selected, and the results were analyzed by using the Ivison-Mac imaging software. The data are expressed as the mean percentage of the control \pm SEM. The value with a letter is significantly different from that of the control, a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

Cellular mechanism studies showed that tanshinones inhibited the growth of prostate cancer cells in part via cell cycle arrest. Interestingly, CT and T1 arrested the cell cycle progression at S phase, whereas T2A did it at G2-M phases, in part via downregulation of cdc2 expression levels (Fig. 2b, 2d). Cdc2, also known as CDK1 (cyclin dependent kinase 1), plays an important role during the cell cycle progress. CDK1 usually combines with Cyclin B and regulates the cell cycle progression at

the S and G2-M phases. CDK1 phosphorylates motor proteins involved in centrosomes separation required for bipolar spindle assembly,⁴¹ and phosphorylates lamina inducing a destabilization of the nuclear structure leading to nuclear envelope breaks down.⁴² It also phosphorylates condensin contributing to chromosome condensation.⁴³ Cdc2 has been considered as an essential molecular target for design of therapeutic anti-cancer drugs.⁴⁴ Therefore, downregulation of cdc2 may provide an

important molecular mechanism that tanshinones modulate cell cycle progression of prostate cancer cells. Further investigation is needed to determine the mechanism by which tanshinones downregulate cdc2 levels. Since Aurora A is essential for G2-M progression^{22,45} and knockdown of Aurora A results in G2-M arrest and eventual apoptosis,⁴⁶ it is possible that the observed cell cycle arrest and downregulation of cdc2 by tanshinones may be, at least in part, due to downregulation of Aurora A. Indeed, Aurora A knockdown reduced cdk1 expression in human carcinoma cells.⁴⁷

Although we found that tanshinones downregulated cdc2 and Aurora A expression in prostate cancer cell lines, our results could not explain the different effects of tanshinones on regulating cell cycle phases, but instead suggested that these tanshinones might have other mechanisms on regulating cell cycle progression. Several regulatory markers, such as cyclin A, cyclin D, cyclin E, CDK2 and others, play roles in S-phase cell cycle progression. It is thus possible that tanshinones CT and T1, but not T2A, may specifically regulate the expression and function of S phase related biomarkers. This will be one of the future mechanistic studies.

Another cellular mechanism by which tanshinones inhibited the growth of prostate cancer cells might be via induction of apoptosis in prostate cancer cells. T1 showed the potent activity in inducing apoptosis of prostate cancer cells (Fig. 2a) in part via downregulation of Bcl-2 and upregulation of Bax levels *in vitro* (Fig. 2b, 2c). T1 also significantly induced apoptosis of DU145 tumor cells *in vivo* (Fig. 6). On the other hand, CT and T2A induced apoptosis of prostate cancer cells primarily via downregulation of Bcl-2 (Fig. 2b, 2c). All treatment significantly increased Bax/Bcl-2 ratio, a more reliable indicator for apoptosis.⁴⁸ Our results are con-

sistent with that of previous *in vitro* studies showing that apoptosis induction was an important cellular mechanism of tanshinone actions in inhibiting the cell growth of different cancer types.^{8,11,14} We further provided the *in vivo* evidence to support that tanshinones induced apoptosis of prostate cancer cells.

The growth of all solid tumors is dependent on angiogenesis and suppression of tumor blood vessel offers a new option for the prevention and treatment of cancer.⁴⁹ Our *in vitro* and *in vivo* studies also provided the convincing experimental evidence to support that one of the mechanisms by which tanshinones, especially T1 inhibited the growth of prostate cancer is by inhibition of angiogenesis. Among tanshinones, T1 showed the most potent anti-angiogenesis activity *in vitro* (Fig. 4) and significantly inhibited prostate tumor angiogenesis *in vivo* (Fig. 6). On the other hand, the molecular mechanisms that tanshinones inhibit angiogenesis remain unclear and should be the important area of research in the future.

In conclusion, the results from this study provided promising experimental evidence to support that T1 may be a novel efficacious and safe candidate agent for the chemoprevention and/or therapy of prostate cancer progression by induction of apoptosis and inhibition of proliferation of prostate cancer cells, and inhibition of prostate tumor angiogenesis. Our results also provided functional evidence to support that Aurora A may be a novel molecular target for tanshinones.

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