

Cryptotanshinone targets tumor-initiating cells through down-regulation of stemness genes expression

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Abstract. Recent evidence indicates that tumor-initiating cells (TICs), also called cancer stem cells (CSCs), are responsible for tumor initiation and progression, therefore representing an important cell population that may be used as a target for the development of future anticancer therapies. In the present study, Cryptotanshinone (CT), a traditional Chinese herbal medicine, was demonstrated to regulate the behaviors of LNCaP prostate cells and prostate LNCaP TICs. The results demonstrate that treatment with CT alters cellular proliferation, cell cycle status, migration, viability, colony formation and notably, sphere formation and down-regulation of stemness genes (Nanog, OCT4, SOX2, β -catenin, CXCR4) in TICs. The present study demonstrates that CT targets the LNCaP CD44⁺CD24⁻ population that is representative of prostate TICs and also affects total LNCaP cells as well via down-regulation of stemness genes. The strong effect with which CT has on prostate TICs suggests that CT may potentially function as a novel natural anticancer agent that specifically targets TICs.

Introduction

The recent identification of cancer stem cells (CSCs) or tumor-initiating cells (TICs) in multiple types of human cancer provides a novel inroad to understanding tumorigenesis at the cellular level (1). Recent evidence supports the hypothesis that the TIC population is responsible for tumor initiation and that

TIC are defined by their ability to self-renew, differentiate and initiate tumors upon transplantation (2-8) In addition, it has been proposed that current drugs used to target cancer are only capable of targeting the differentiated cancer cells and available treatments have the capability to shrink and de-bulk tumors but are unable to target the TICs, the population responsible for tumor initiation. Unfortunately, this inability of current anti-cancer treatments to target TICs results in the re-establishment of the tumor by the remaining and viable TIC population (9). Hence, the TIC hypothesis provides a novel target for the treatment of cancer.

Prostate cancer is the third leading cause of cancer-associated mortalities among men, behind colon and lung cancer and leads all non-skin cancer malignancies. Prostate cancer is initially treated with androgen deprivation therapy by either surgical castration or medical castration with gonadotropin-releasing hormone agonists (10). However, the response to androgen deprivation therapy in the metastatic setting is transient and tumors progress to castration-resistant prostate cancer, which is marked by a gain-of-function in androgen receptor (AR) and AR reactivation. According to the TIC hypothesis, AR would be expressed in the prostate cancer stem cell since there would be genetic selection for gain-of-function changes in AR, such as AR gene amplification (11). TICs are emerging as being important in prostate cancer metastasis and are coming to the forefront as targets of therapy. The ability to purify TICs and study mechanism(s) which may be utilized to target TICs is very important in the development of future prostate cancer treatment. Hurt *et al* (12) showed that CD44⁺/CD24⁻ cells purified from the LNCaP cell line were more clonogenic, tumorigenic, and invasive than the corresponding depleted cells. Duhagon *et al* (13) demonstrated that TICs can be enriched using a sphere formation assay resulting in the culture of prostatespheres (PSs). Furthermore, Duhagon *et al* (13) provided a genomic profile of PSs that coordinated with the genomic profile of the prostate CD44⁺CD24⁻ TIC population demonstrating that PSs are representative of the TIC population. Klarmann *et al* (14) demonstrated that the invasive cells in the prostate LNCaP cell line are more tumorigenic in NOD/SCID mice compared with noninvasive cells and have a genomic profile similar to CD44⁺CD24⁻ cells as well. Hence, the CD44⁺/CD24⁻ cells,

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the PSs and the invasive cells in prostate cancer cell lines are all representative of prostate TICs. These populations of cells express high levels of stem cell-associated genes, including OCT3/4, BMI, β -catenin, and smoothened (SMO) which is characteristic of TICs. Additionally, TICs appear to be more resistant to conventional chemotherapies and radiation, thereby, contributing to the development of metastatic and resistant disease (9,15). Given these considerations, the present study sought to investigate if prostate TICs can be targeted by Traditional Chinese Medicines (TCM) to result in the prevention of tumor initiation, progression and relapse.

Herbal therapies and products commonly used in TCM are attracting increasing attention in the field of cancer. The principles underlying TCM were established over thousands of years based on clinical experience and practice. In China, the majority of cancer patients use some form of Chinese medicine, including prescription medications and non-prescription medications (16). On a global level, it has been reported that more than half of all cancer patients now use some form of complementary/alternative medicine, yet the majority of these patients do not disclose this use to their physicians (17). There are numerous clinical reports indicating that patients benefit from TCM treatment including Lin *et al* (18), which observed 173 cases of non-small cell lung cancer (NSCLC) patients, post-surgery, with two years of treatment with standard chemoprevention alone or combined with TCM herbs: The result of this study indicated that the relapse and distant metastasis rate of patients in the TCM group was 45.09% and the control group was 50.6%. Yang *et al* (19) evaluated the effectiveness of comprehensive TCM treatment in reducing the relapse and metastasis of stage II and III colorectal cancer based on conventional Western medicine (WM) therapy: In this study, 222 patients were recruited and assigned to two groups based on whether or not they were additionally treated with TCM comprehensive therapy. The relapse/metastasis rate in the combined group at 1-, 2-, 3-, 4-, and 5-years was 0 (0/98), 2.04% (2/98), 11.69% (9/77), 14.06% (9/64), and 21.28% (10/47), respectively (18). In the group given WM, the relapse/metastasis rates were 4.80% (5/104), 16.35% (17/104), 21.65% (21/97), 25.93% (21/81), and 38.18% (21/55), respectively, for 1-, 2-, 3-, 4- and 5-years (19). The median relapse/metastasis time was 26.5 months in the combined group and 16.0 months in the WM group. These two studies provide a strong foundation of evidence that TCM can prohibit the relapse and metastasis of cancer. Additionally, it has been previously shown that TCM therapy can also prevent tumorigenesis (20). Liang *et al* (21) demonstrated that the TCM Liuwei Dihuang Wan, can prohibit progression of the precancerous disease of esophageal cancer. In this specific study, 214 patients with hyperplasia of esophageal epithelial cells were treated with Liu wei Di Huang Wan and after 2 years, the cancerous changes in the Liu wei Di Huang Wan treatment group was 1.4%, but in the placebo group was 6.3% (22).

Unfortunately, the active ingredients in the majority of TCM herbs and their mechanism(s) have not been identified. However, it is clear that TCM is capable of preventing tumorigenesis and both the relapse and metastasis of cancer (23). Previous studies have indicated that certain naturally occurring phytochemicals are cytotoxic to TICs, such as parthenolide (PTL) derived from suayule, can specifically target TICs in

primary human acute myelogenous leukemia (AML) (24). Additional studies demonstrated that PTL has toxicity on both the side population and mammospheres isolated from breast cancer which are representative of TICs, and lastly, Kawasaki *et al* (25) demonstrated that PTL is cytotoxic to prostate TICs. The phytochemical sulphoraphane, derived from broccoli, can inhibit breast cancer TICs and down-regulate the Wnt/ β -catenin self-renewal pathway (26). Gossypol, a bioactive phytochemical produced by cotton plants, was effective at inhibiting prostate tumor-initiating cell-driven tumor growth in a NOD/SCID xenograft model (27).

Current evidence suggests that TICs are responsible for tumorigenesis, relapse and metastasis (9) and can be targeted using naturally occurring compounds, hence, the present study aimed to determine whether components of TCM medicine, which are often phytochemicals, could inhibit or eradicate prostate TICs as well. Cryptotanshinone (CT), an active component of the Danshen root, is a popular TCM herb used in the clinic to treat chronic hepatitis, coronary heart disease (28,29) and cancers such as hepatic cancer (30) and leukemia (31). The evidence indicates that CT has biological effects ranging from anti-inflammatory, -bacterial, -fibrotic, -oxidative, -mutagenic, and -platelet aggregation activities (29,32-34). There are a few reports on the antitumor effect of CT, which include *in vitro* studies from Gong *et al* (35) that demonstrated that CT can inhibit the growth of human prostate cancer cell lines in a dose-dependent manner via cell cycle arrest and induction of apoptosis. In addition, Park *et al* (36) demonstrated that CT can suppress Bcl-2 expression and augment Fas sensitivity in DU145 cells. Further work indicates that JNK and p38 MAPK act upstream of Bcl-2 in Fas-treated DU145 cells and that CT can significantly block activation of these kinases (36). Shin *et al* (37) showed that CT is a potent anti-cancer agent and has antitumor activity through the inhibition of STAT3. Notably, biological behaviors such as cell migration and invasion are related to TICs, and there are reports indicating that CT has the ability to block invasion of bovine aortic endothelial cells induced by bovine fibroblast growth factor (bFGF) (38) and could function in inhibiting leukocyte chemotactic migration (39). Taken together, the present study hypothesized that CT has the potential to target prostate TICs and investigated if CT was capable of targeting TICs derived from the LNCaP prostate cell line. The results provide evidence that CT can target TICs and selectively inhibit their proliferation. Furthermore, the present study presents data that indicates CT driven inhibition of TICs occurs by regulating the expression of stemness genes that are associated with the self renewing ability of TICs. Based on this data, CT, an important TCM compound, has a potential effect in targeting TICs and it may provide an alternative means of treatment and options in the investigation for TIC targeting drugs.

Materials and methods

Cells and media. LNCaP cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin and streptomycin (all, Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Assay of cell viability. Cells were plated at a density of 1,000 cells per well in a 96-well plate and viability was measured using Cell-Titer Glo assay (Promega Corporation, Madison, WI, USA). The Cell-Titer Glo reagent was added to each well and equilibrated for 30 min before measurements were taken. Luminescence was measured using an Infinite M200 plate reader (Tecan Group, Ltd., Männedorf, Switzerland).

Flow cytometric analysis and cell sorting. LNCaP cells were detached with trypsin (Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA), washed once in FACs buffer [PBS containing 1-2% bovine serum albumin (BSA; Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA)], then stained with 5 μ l of Invitrogen anti-CD24-FITC (mouse monoclonal; catalog no., MHCD2401-4; Thermo Fisher Scientific, Inc.) and 0.5 μ l of antibody per 10^6 cells of anti-CD44-PE (mouse monoclonal; catalog no., 12-0441-82; eBioscience, Inc., San Diego, CA, USA) and incubated at 4°C for 15 min. Following incubation, cells were washed twice with FACs buffer. For flow cytometric sorting, cells were re-suspended in FACs buffer at 20×10^6 cells/ml and separated on an Aria cell sorter (BD Biosciences, San Jose, CA, USA). Live cells were gated on the basis of forward and side scatter, and single cells were gated on the basis of forward scatter and pulse width. Gates were determined by analysis of unstained cells, isotype specific stains, and single stains (WinMDI version 2.9; BD Biosciences). The CD44⁺CD24⁻ cells were not assessed for purity due to the low numbers of cells obtained.

Apoptosis assay. LNCaP cells were treated with or without varying concentrations of CT (0, 2.5, 5 and 10 μ M). After 48 h, cells were collected and a quantitative apoptotic death assay was performed using Annexin V and propidium iodide (PI) staining. After trypsinization, the cells were re-suspended in the binding buffer with FITC-conjugated Annexin V and PI (BD Biosciences) for 20 min at room temperature in the dark. All samples were then FACS analyzed on an Aria cell sorter as described above to distinguish early-apoptotic cells, late-apoptotic and necrotic cells.

Cell cycle analysis. In total, 1×10^4 cells were seeded and treated with or without CT (catalog no., 10852-200806; National Institutes for Food and Drug Control, Beijing, China) at different doses according to the results of the cell viability assay (0, 2.5, 5 and 10 μ M). Following treatment, 1×10^6 cells were fixed in ice cold 70% ethanol overnight. Following fixation, cells were centrifuged (5 min; $100 \times g$) and re-suspended in PBS containing 40 μ g/ml propidium iodide and 100 μ g/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 1 h. Samples were then FACS analyzed on an Aria cell sorter as described above and cell cycle analysis was performed.

Wound healing. The cells were seeded in a 12-well tissue culture dish at a concentration of 1×10^5 cells/well and maintained in RPMI-1640. After the cells reached 80-90% confluency, the tip of a micropipette was used to wound the cells, creating a linear and cross-stripe scrape 2 mm apart. The cells were washed with PBS to remove floating cellular debris and re-fed for an additional 24 h with or without CT treatment (1.25, 2.50 and

5.00 μ M). Images of wound closure or cell migration were captured when the scrape wound was introduced and 24 h after wounding, using an IX70 inverted microscope equipped with a digital camera (Olympus Corporation, Tokyo, Japan).

Soft agar colony assay. Cells were seeded at a concentration of 1,000 cells and suspended in RPMI-1640+10% FBS containing 0.6% agarose (Sigma-Aldrich) and overlaid onto a 12-well plate containing a solidified bottom layer of RPMI-1640+10% FBS plus agarose. Once the top layer solidified, 200 μ l of medium was placed on top to keep the plates moist. The plates were incubated for 2 weeks until colonies were visible. The plates were stained with 5 mM MTT (Sigma-Aldrich) at 37°C for 1 h, then counted and imaged by using GelCount™ automatic plate scanner (Oxford Optronics Ltd., Abingdon, UK) and GelCount Version 0.025.1 software (Oxford Optronics).

Sphere formation assay. In order to obtain prostatospheres from LNCaP cells, the exponentially growing cultures were dissociated to single cells by standard trypsinization, washed three times with PBS and plated in stem cell medium [SCM; Dulbecco's Modified Eagle Medium F12 (Gibco®; Thermo Fisher Scientific, Inc.), 10 ng/ml bFGF (Sigma-Aldrich), 20 ng/ml endothelial growth factor (Sigma-Aldrich), 5 mg/ml insulin (Sigma-Aldrich), and 0.4% BSA] supplemented with 1% KO serum replacement (Invitrogen; Thermo Fisher Scientific, Inc.) at a density of 1,000 cells/ml in tissue culture treated flasks. After approximately 7 days, spheres were counted and analyzed using a GelCount™ automatic plate scanner (Oxford Optronics) and GelCount Version 0.025.1 software (Oxford Optronics).

Gene expression analysis. Total RNA isolation was performed using TRIzol reagent (Gibco®; Thermo Fisher Scientific, Inc.) on untreated LNCaP cells, CT-treated LNCaP cells, untreated CSCs cells that were enriched from LNCaP cells and CT-treated CSCs cells that were enriched from LNCaP cells. cDNA was synthesized with Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Inc.) using random hexamers and following the manufacturer's instructions. Analysis of gene expression by reverse transcription quantitative-polymerase chain reaction (RT-qPCR) was performed using TaqMan™ Gene Expression assays (Nanog assay ID, Hs04399610_g1; SOX-2 assay ID, Hs00367969_m1; OCT4 assay ID, Hs00999632_g1; BMI1 assay ID, Hs00995536_m1; MMP9 assay ID, Hs00234579_m1; NF κ B1 assay ID, Hs00765730_m1; β -catenin assay ID, Hs00355049_m1; CXCR4 assay ID, Hs00607978_s1; β -actin assay ID, Hs01060665_g1; Thermo Fisher Scientific, Inc.) in a StepOne Real-Time PCR machine (Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 10 min at 95°C; and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative mRNA expression levels were calculated as $2^{-\Delta\Delta C_t}$ and were normalized against β -actin (40). The experiment was repeated three times.

Western blot analysis. Total protein was isolated from LNCaP cells using RIPA lysis buffer (Thermo Fisher Scientific Inc.) and quantified using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). A total of 20 μ g of

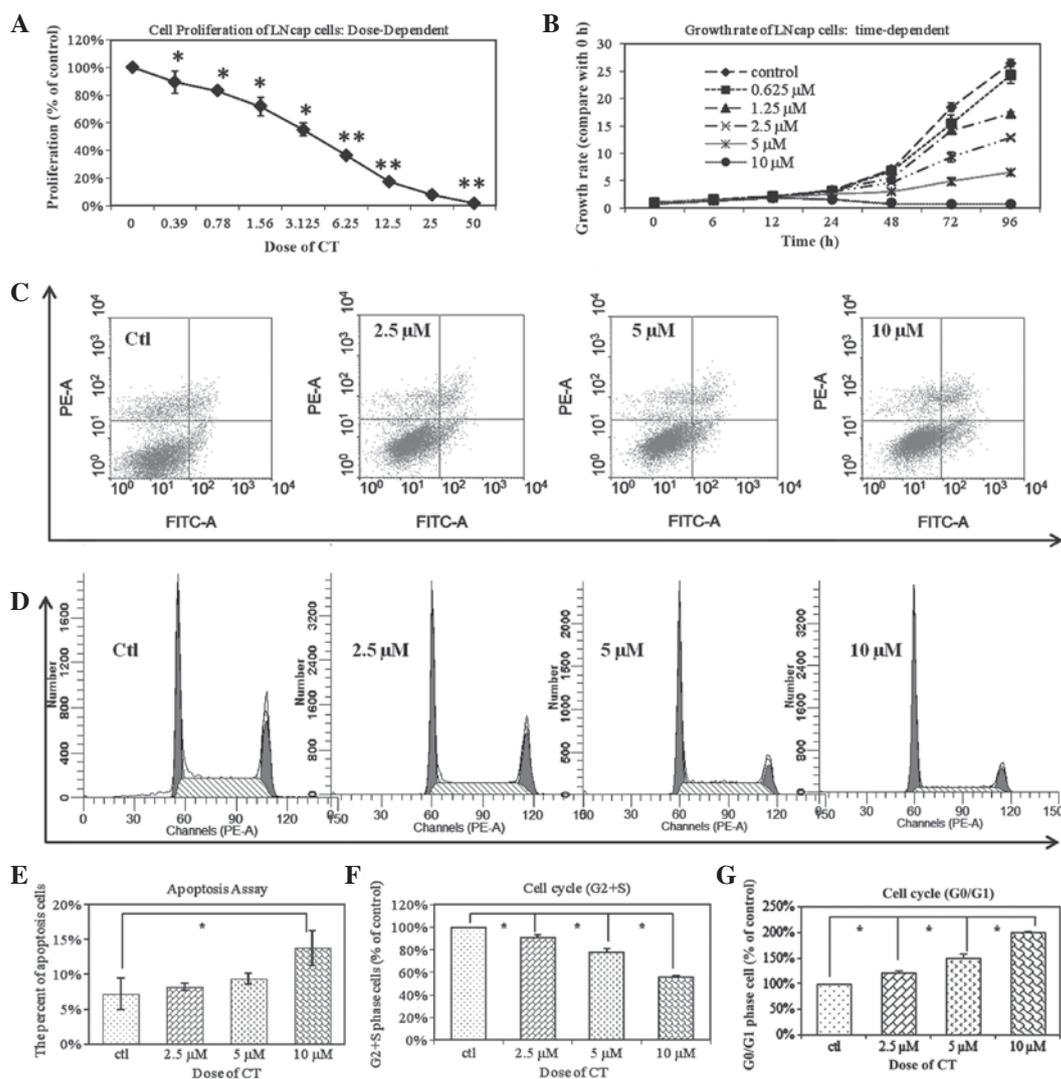


Figure 1. CT changes the biological behaviors of total LNCaP cells. (A) LNCaP cells were treated with an increasing dose of CT ranging from 0.39 to 50 μM for 48 h. CT inhibited the proliferation of total LNCaP cells in a dose-dependent manner ($P < 0.05$; $^{**}P < 0.01$). (B) LNCaP cells were observed over 6–96 h and treated with 0–10 μM CT as indicated. The IC_{50} of CT is 5 μM ; inhibition was time-dependent and greatest after 8 h. (C) LNCaP cells were treated with increasing doses of CT (2.5 μM , 5 μM and 10 μM). FACS analysis demonstrated that CT induced the cell apoptosis of Total LNCaP cell in a dose-dependent manner. (D) FACS analysis demonstrated that treatment with CT results in LNCaP cell cycle arrest, in a dose-dependent manner. (E) Statistical analysis of apoptosis demonstrates there is a significant difference between the control group and the 10 μM CT treated groups ($P < 0.01$). (F) The relative percentages of CT treated LNCaP cells in the S and G2/S phases of the cell cycle. The percentage of CT treated cells distributed at the G2/S phase was significantly reduced in a dose-dependent manner from 69.73 to 63.5, 54.25 to 39.05% after 48 h of treatment with 2.5, 5 and 10 μM CT, respectively ($P < 0.01$). (G) A total of 36.55%, 45.75%, and 60.95% of LNCaP cells were distributed at the G0/G1 phase after 48 h of treatment with 2.5, 5 and 10 μM CT, respectively. This was in comparison to the 30.27% of untreated cells in G0/G1 phase ($P < 0.01$; $^{**}P < 0.05$). FACS, fluorescent activated cell sorting; CT, cryptotanshinone.

protein extract was loaded per lane into a 4–20% Tris-glycine gel (Invitrogen; Thermo Fisher Scientific Inc.), transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA), blocked in 5% BSA and incubated with the following primary polyclonal rabbit antibodies from Abcam (Cambridge, UK): Anti-Nanog (catalog no., ab80892; dilution, 1:1,000); anti-actin (catalog no., ab194952; dilution, 1:10,000); anti-CXCR4 (catalog no., ab93478; dilution, 1:1,000); anti- β -catenin (catalog no., ab6302; dilution, 1:5,000); anti-SOX2 (catalog no., ab97959; dilution, 1:1,000); and anti-OCT4 (catalog no., ab125949; dilution, 1:1,000). All secondary antibodies obtained from LI-COR (IRDye[®] goat anti-rabbit IgG; catalog no., 926-32221; LI-COR Biosciences, Lincoln, NE, USA). Blots were scanned using the LI-COR Odyssey IR Imaging System.

Statistical analysis. Unless specified, all experiments were performed in triplicate and were repeated at least twice. Data is expressed as mean values, standard error of the mean or standard deviation and were analyzed by analysis of variance. SPSS version 10.0 (SPSS Inc., Chicago, IL, USA) was used for analysis. The level of significance was set at $P < 0.05$.

Results

The anti-proliferative effects of CT on LNCaP cells. To determine whether CT inhibited cellular proliferation of the LNCaP line, cells were treated with increasing concentrations of CT as indicated and cell viability was assessed. Fig. 1A demonstrates that CT inhibited cellular proliferation at 0.39 to 50 μM doses for 48 h. Fig. 1B demonstrates that CT inhibition of LNCaP

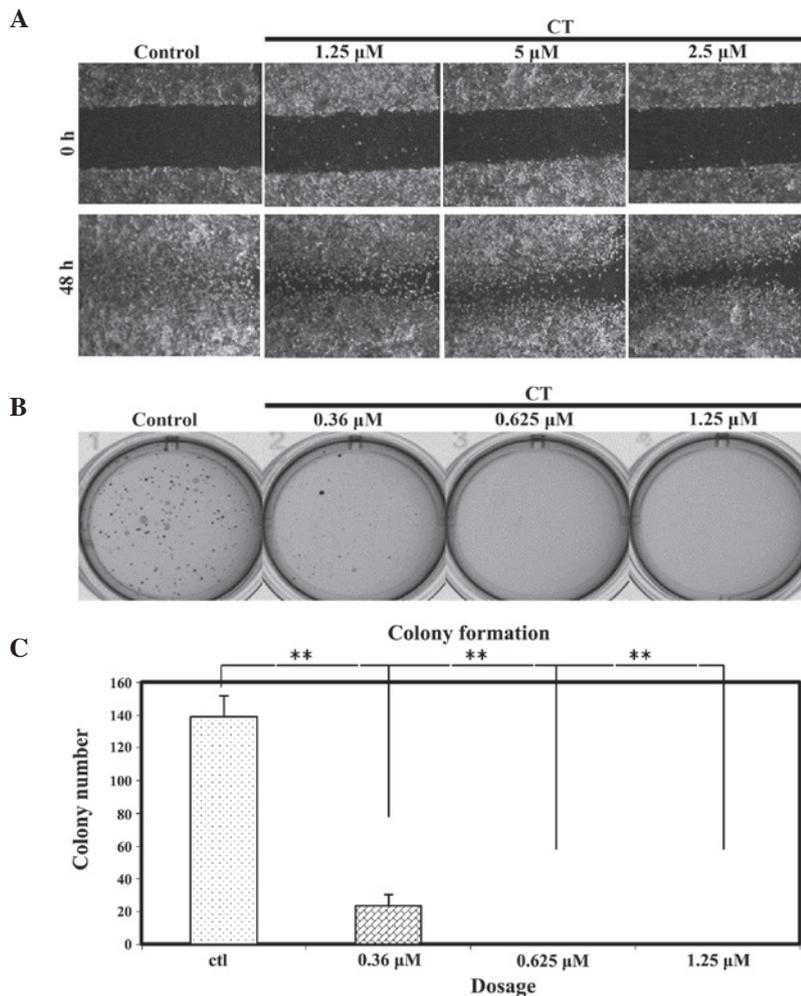


Figure 2. CT inhibits the wound healing and the colony formation behaviors of total LNCaP cells: (A) Wound healing assay was performed on LNCaP cells using increasing doses of CT. For the untreated cells, migration occurred and the wound was healed in 48 h; however, in cells treated with CT, cell migration was partially inhibited. (B) Colony formation efficiency of LNCaP cells was inhibited by a serial dilution of CT and (C) quantification revealed the difference to be statistically significant (**P<0.01). Data from a representative experiment (from a total of three) are shown. CT, cryptotanshinone.

proliferation is time-dependent as a time period of 6-96 h was tested at various doses as indicated. The IC₅₀ of CT is 5 μM and is most effective after 48 h. The anti-proliferative effect of CT on LNCaP cells was a dose and time-dependent. Based on these results, the subsequent experiments were performed with a dose of 5 μM and the time period the cells are treated with CT varies based on the experiment performed and is indicated.

To further determine whether the inhibition in proliferation by CT is due to an induction in apoptosis, LNCaP cells were treated with CT and analyzed apoptosis using FACS analysis. Fig. 1C demonstrates that CT has an apoptotic effect on LNCaP cells and this effect is dose dependent. However, there was a significant difference among the control group and the 10 μM CT treated groups (Fig. 1E). Based on this observation, the anti-proliferative effects were analyzed of CT was due to perturbation of the cell cycle. LNCaP cells were treated with different concentrations of CT: The number of CT treated LNCaP cells distributed at the G2/S phase after 48 h of treatment with 2.5, 5 and 10 μM CT was also significantly decreased from 69.73 to 63.51%, 54.25%, and 39.05%, respectively in a dose dependent manner (Fig. 1F; P<0.01). A total of 36.55, 45.75 and 60.95% of the cells were distributed at the

G0/G1 phase after 48 h of treatment with 2.5, 5 and 10 μM CT, respectively; this was in comparison to the 30.27% of untreated cells in G0/G1 (Fig. 1G; P<0.01). This data indicates that CT inhibits cellular proliferation by inducing G0/G1 phase arrest in cancer cells.

CT affects biological behaviors of total LNCaP cells. To determine if CT could affect specific biological behaviors of LNCaP cells such as the ability to migrate, a wound healing assay was performed on LNCaP cells treated with CT (Fig. 2A). After making a wound, LNCaP cells were treated with various concentrations of CT, as indicated, and then the wound was inspected microscopically over time as the cells migrated to fill the damaged area. For the untreated cells, migration occurred and the wound was healed in 48 h; however, in cells treated with CT, cell migration was partially inhibited. The data suggests that the level of migration was affected in a dose dependent manner.

An additional characteristic of cancer cells that affects their biological behavior is the ability to form colonies in anchorage independent conditions. The present study investigated whether CT has the ability to affect colony formation.

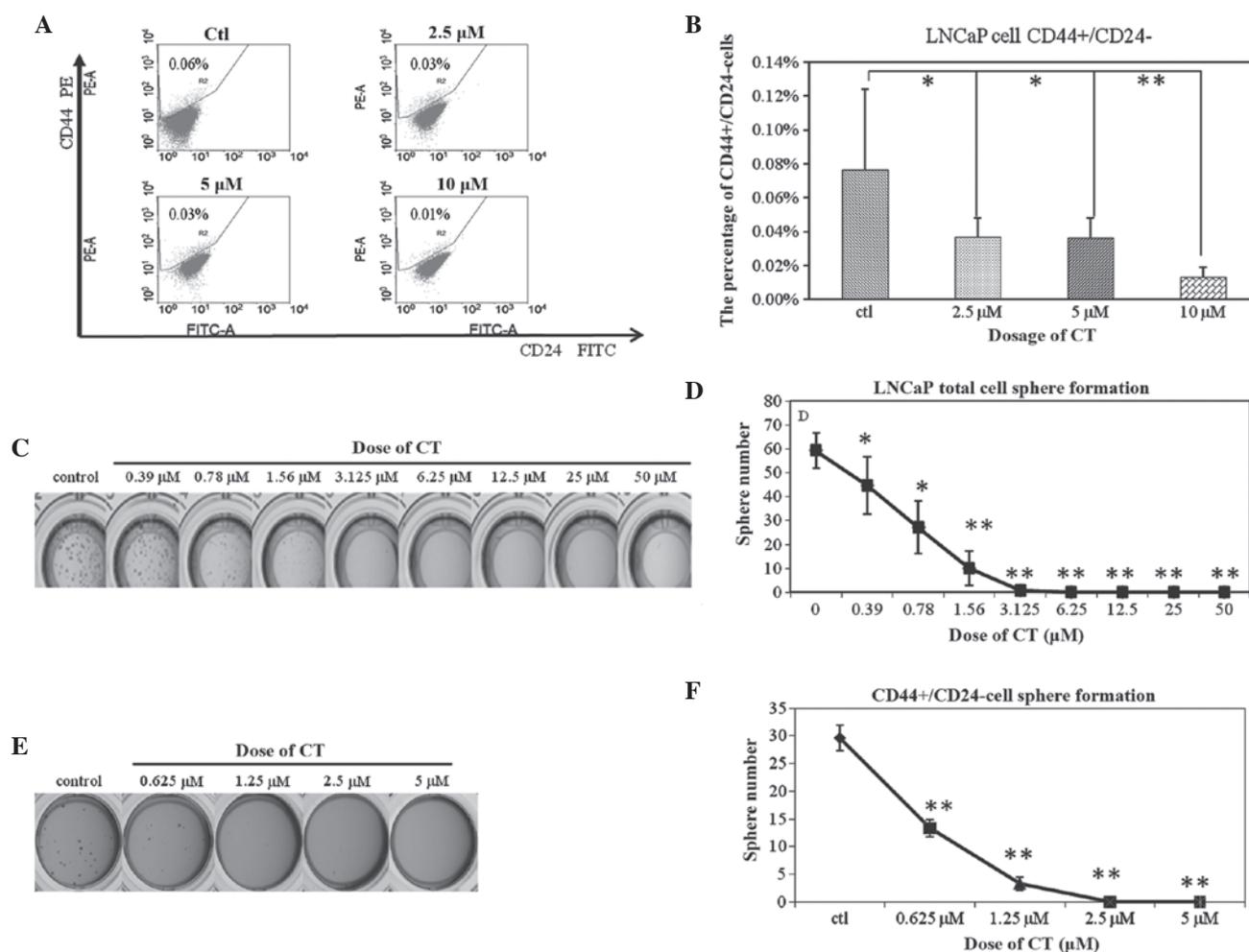


Figure 3. CT targets LNCaP TICs. (A) FACS analysis was performed to determine the percentage of CD44⁺CD24⁻ cells after treatment with increasing doses of CT (2.5, 5 and 10 μM). Data from a representative experiment (from a total of three) are shown. (B) Percentage of CD44⁺CD24⁻ LNCaP cells compared to untreated control. The percentage reduced in a dose dependent manner, however no statistically significant difference was observed between the control and CT treated groups. (C) Sphere formation assay quantification was performed using increasing doses of CT. (D) The sphere forming ability was significantly inhibited by CT while the dose up to 1.5 μM, (*P<0.05; **P<0.01). (E) Sphere formation assay of CD44⁺/CD24⁻ cells was performed using increasing doses of CT. (F) The sphere forming ability were inhibited by CT while the dose up to 0.625 μM (**P<0.05; *P<0.01). FACS, fluorescent activated cell sorting; CT, cryptotanshinone; TICs, tumor initiating cells.

Fig. 2B and C demonstrates that the colony formation efficiency of LNCaP cells was significantly inhibited by CT at very low doses (0.36, 0.625 and 1.25 μM). Notably, at the IC₅₀ concentration of 5 μM, colony formation did not occur (data not shown); therefore, a serial dilution of CT was performed and it was observed that colonies do not form following CT treatment when the dose is as low as 0.625 μM.

CT affects the biological behaviors of LNCaP TICs. Based on the above data, CT has the ability to inhibit cellular proliferation of LNCaP cells in a time and dose dependent manner; reduced the percentage of cells which are in the proliferative phase of the cell cycle; prolonged and partially inhibited the ability to migrate after cell wound healing; induced apoptosis of LNCaP cells and lastly, inhibited colony formation in soft agar. As previously stated, there are various phenotypes associated with prostate TICs such as cell migration and soft agar colony formation (13,14). Hence, we sought to investigate if CT has the ability to specifically regulate the biological behaviors of LNCaP TICs.

Our laboratory has previously shown that the CD44⁺CD24⁻ cell population is the TIC population in the LNCaP cell line

and the present study sought to investigate if 48 h CT treatment would alter the percentage of CD44⁺CD24⁻ cells (14). A dose-dependent decrease of this subpopulation was observed (Fig. 3A); 0.08±0.05%, 0.04±0.01%, 0.04±0.01% and 0.01±0.01% of the total cells reflect the CD44⁺/CD24⁻ population after 48 h of treatment with 0, 2.5, 5 and 10 μM CT, respectively. However, these results were not statistically significant (Fig. 3B). Based on these observations, this decrease may be a result of CT functioning as an inhibitor of TIC viability, TIC proliferation or as an inducer of differentiation, resulting in an overall reduction of the TIC population.

Previously our laboratory has shown that the sphere formation assay can enrich for TICs of LNCaP cells (13), hence, the total cells were cultured in highly defined stem cell media as previously described and cultured with or without CT. The results demonstrate that CT inhibit sphere formation of LNCaP cells at a dose of 1.5 μM (Fig. 3C and D), a dose previously shown to have little effect on total LNCaP viability (less than 30% Fig. 1A). Based on these observations, it was hypothesized that CT could specifically inhibit the proliferation or self-renewing ability of TICs. To confirm whether CT could

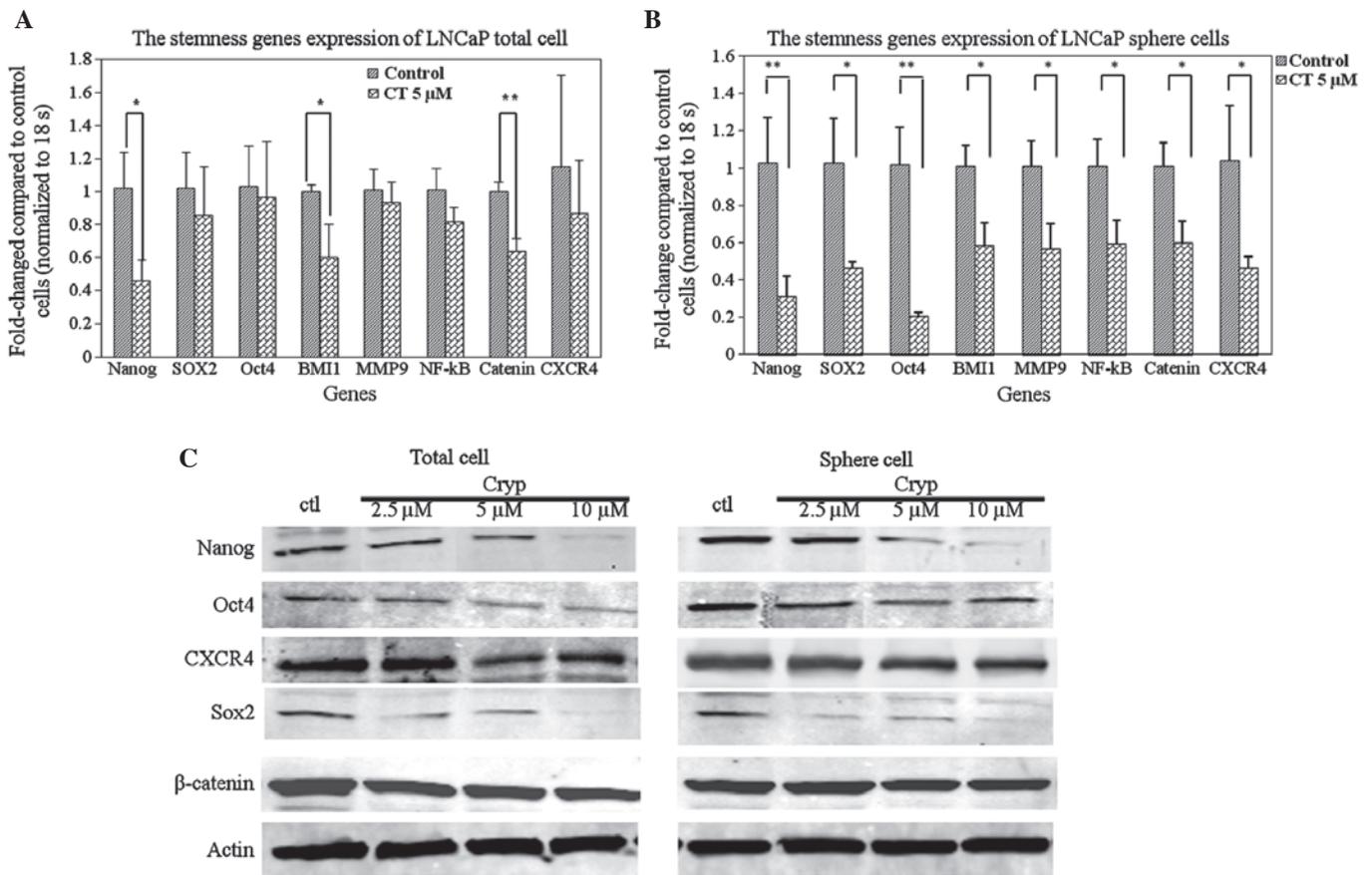


Figure 4. CT affects mRNA and protein levels of stemness genes in both total and TIC LNCaP cells. (A) Total LNCaP cells were treated with CT for 24 h and RT-qPCR was performed. Statistically significant reductions in the mRNA expression of Nanog, BMI1 and Catenin, were observed (* $P < 0.05$; ** $P < 0.01$). (B) LNCaP spheres were treated with CT for 24 h and RT-qPCR was performed (* $P < 0.05$). Data is normalized to 18srRNA. (C) Western blot analysis was performed on both total and sphere LNCaP cells to determine expression levels of Nanog, OCT4, Sox2, β -catenin and CXCR4. The left volume are Total LNCaP cell and the right volume are sphere cells. Data from a representative experiment (from a total of three) are shown. CT, cryptotanshinone; TICs, tumor initiating cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

indeed inhibit TIC self-renewal, the LNCaP CD44⁺CD24⁻ population was isolated by FACs sorting and cultured in SCM with or without CT. As shown in Fig. 3E and F, the ability to form spheres was decreased from 29.99 ± 2.3 in the control group to 13.3 ± 1.52 in the $0.625 \mu\text{M}$ CT group, 3.33 ± 1.15 in the $1.25 \mu\text{M}$ CT group, and 0 in the $2.5 \mu\text{M}$ and $5 \mu\text{M}$ CT group. Based on these data, evidence is provided that CT inhibits the sphere forming ability of CD44⁺CD24⁻ cells. This evidence supports the hypothesis that CT can target the self-renewing ability of TICs.

CT regulates stemness genes associated with LNCaP TICs.

To further understand the possible molecular mechanisms by which CT can regulate LNCaP TIC self-renewal, the present study investigated whether CT can inhibit the expression of TIC related genes implicated in self-renewal by RT-qPCR and western blot analysis. Nanog, SOX2 and Oct3/4 genes are important transcription factors orchestrating the self-renewal of stem cells (41); therefore, these factors and additional stem associated factors were selected. As shown in Fig. 4A, it was demonstrated that CT treatment reduced the expression levels of certain stemness genes in total cells, specifically the expression of Nanog, BMI1 and β -catenin: A statistically significant decrease of mRNA levels was observed between

the control and $5 \mu\text{M}$ CT treated groups ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively). To further investigate the hypothesis that CT specifically affects TICs by decreasing the expression of stemness genes, RT-qPCR was performed on LNCaP cell spheres treated with or without CT. Fig. 4B demonstrates that treatment with CT resulted in a decrease of expression of stemness genes in LNCaP spheres. The decrease in expression was statistically significant in all the detected genes ($P < 0.01$). Lastly, western blot analyses confirmed the observations in the RT-qPCR. CT treatment reduced Nanog, SOX2 and OCT4 protein expression levels in a dose-dependent manner in total (Fig. 4C, left) and sphere cells (Fig. 4C, right).

The Wnt signaling pathway is important in embryogenesis (42), and is also essential for the self-renewal of stem cells by preventing cellular differentiation. Our data shows that β -catenin expression is down-regulated by CT (Fig. 4C) and further confirms that CT affects important regulatory factors involved in self-renewal of prostate TICs. The survival and maintenance of stem cells requires a balance between the processes of both self-renewal and differentiation. This is hypothesized to be regulated by a niche or protective micro-environment that is required to anchor the stem cells (43,44). CXCR4, a G-protein coupled receptor, has recently been confirmed as a TIC marker as well and is expressed at a

significantly higher level in this population compared to the non-TICs (45-48). To determine if CT could affect CXCR4 expression, the expression levels of CXCR4 were analyzed in LNCaP TICs at the mRNA and protein level. The results indicated that CXCR4 is down-regulated at the mRNA and protein levels in the total and TIC population (Fig. 4). Based on the ability of CT to regulate stem associated genes and CXCR4, it is possible that CT also regulates biological behaviors associated with the function of the SDF1/CXCR4 axis such as migration and metastasis.

Discussion

Our previous work indicates that both sphere forming and CD44⁺CD24⁻ populations in LNCaP cells reflect a population with a capacity to undergo self-renewal and initiate tumor formation which are major properties of TICs (12,13). An additional characteristic of TICs is the upregulation and increased expression of stem associated transcription factors such as Nanog, SOX2 and OCT4, which are found to modulate embryonic stem cell self-renewal (41). The evidence supporting the aggressiveness and ability of these cells to initiate tumors and function in metastasis has led to the study of these TICs as an important potential target for cancer therapy. The present study provides several lines of evidence that CT has the ability to inhibit the self-renewing ability of prostate TICs. The present study demonstrated that CT treatment reduced the percentage of CD44⁺CD24⁻ prostate cancer cells (Fig. 3A) and reduced the number of LNCaP derived prostatospheres (Fig. 3B and C). Lastly, it was demonstrated that CT can regulate several genes known to regulate LNCaP TICs, including SOX2, Nanog and OCT4 which are critical transcription factors in the regulation of both ES and TIC cell properties (38) (Fig. 4). Collectively, this data indicates that CT can reduce and effect the TIC population.

As recent studies have shown, Nanog, SOX2 and OCT4 serve crucial roles in stem cell self-renewal. These coordinated transcription factor networks involving OCT4, SOX2, and Nanog have currently emerged as the master regulatory mechanisms of stem cell self-renewal and differentiation (49). It has been shown that knockdown of *OCT4* can induce ES cells to differentiate into trophectoderm-like cells (50) and knockdown of *SOX2* results in ES cell differentiation (51,52). MicroRNAs responsible for the regulation of *OCT4*, *SOX2*, and *Nanog* coding regions are found to modulate embryonic stem cell differentiation as well (41). Notably, miR-302 which can target OCT4/SOX2/Nanog has been shown to have a role in converting differentiated cells to induced pluripotent stem cells (53,54). However, their dysfunction in cancer may contribute to the maintenance of an undifferentiated proliferative phenotype by preventing the expression of differentiation genes and allowing the expression of genes promoting stem cell renewal (55,56). Further evidence supporting the critical role of OCT4, SOX2, Nanog and Lin28 in stem cells were seen when transfection of these factors in 293 FT cells demonstrated an impaired ability to differentiate and form immature ectodermal tumors after they were transplanted into nude mice (57). In a lung adenocarcinoma model, OCT4 and Nanog are highly expressed in CD133⁺ but not in the CD133⁻ population (58) and in a glioblastoma model, Nanog appears to be

critical in the ability of undifferentiated stem cells to undergo self-renewal and is the most differentially expressed (59). The ability of CT to inhibit OCT4, SOX2, Nanog by CT confirms that CT can target the self-renewal ability of prostate TICs.

Another important gene in TIC regulation is β -catenin and our data further indicates that CT can down-regulate expression of β -catenin as well. The Wnt signaling pathway is essential for the maintenance of the majority of tissue stem cell compartments by preventing cellular differentiation such as seen in intestinal and hematopoietic stem cells (60). β -catenin is a key protein in the regulation of this pathway, therefore, inhibition of β -catenin by CT further confirms that CT can target the self-renewal ability of prostate TICs.

In order for stem cells to survive and maintain the balance between self-renewal and differentiation, a niche or protective microenvironment is required to anchor the stem cells (43,44,61) CXCR4 is a G-protein coupled receptor that is expressed constitutively in a wide variety of normal tissues, including lymphatic tissues, thymus, brain, spleen, stomach, and small intestine (62). Within the microenvironment, both stromal derived factor (SDF-1) and its receptor CXCR4 is essential for the cell anchoring process (15,63). It is well documented that disseminated prostate cancer cells express CXCR4 and can home to sites where SDF is present such as the bone and lymph nodes (63). Recently, CXCR4 was detected on TICs and mRNA levels were found to be significantly higher in this population in comparison to the non-TICs (44-48). The upregulation of CXCR4 cell surface expression corresponded to a significant increase in EMT in response to SDF1- α *in vitro* (64,65). In the present study, CT was shown to down-regulate CXCR4 expression in total LNCaP cells and the TIC population; hence, it is possible CT can further regulate TICs by regulating the CXCR4-SDF1 axis and disrupting its ability to interact with the microenvironment. However, further work needs to be performed to confirm this.

In conclusion, this is the first report demonstrating a role for CT as a regulator and inhibitor of prostate TICs. CT can specifically inhibit the self-renewal of TICs by targeting key transcriptional regulators. The data demonstrating that CT can regulate these factors indicates that CT has the potential to function as a natural anti-cancer agent targeting prostate TICs.

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