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Cryptotanshinone suppresses androgen receptor-mediated growth in androgen dependent and castration resistant prostate cancer cells

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ABSTRACT

Androgen receptor (AR) is the major therapeutic target for the treatment of prostate cancer (PCa). Anti-androgens to reduce or prevent androgens binding to AR are widely used to suppress AR-mediated PCa growth; however, the androgen depletion therapy is only effective for a short period of time. Here we found a natural product/Chinese herbal medicine cryptotanshinone (CTS), with a structure similar to dihydrotestosterone (DHT), can effectively inhibit the DHT-induced AR transactivation and prostate cancer cell growth. Our results indicated that 0.5 μM CTS effectively suppresses the growth of AR-positive PCa cells, but has little effect on AR negative PC-3 cells and non-malignant prostate epithelial cells. Furthermore, our data indicated that CTS could modulate AR transactivation and suppress the DHT-mediated AR target genes (PSA, TMPRSS2, and TMEPA1) expression in both androgen responsive PCa LNCaP cells and castration resistant CWR22rv1 cells. Importantly, CTS selectively inhibits AR without repressing the activities of other nuclear receptors, including ERα, GR, and PR. The mechanistic studies indicate that CTS functions as an AR inhibitor to suppress androgen/AR-mediated cell growth and PSA expression by blocking AR dimerization and the AR–coregulator complex formation. Furthermore, we showed that CTS effectively inhibits CWR22Rv1 cell growth and expressions of AR target genes in the xenograft animal model. The previously un-described mechanisms of CTS may explain how CTS inhibits the growth of PCa cells and help us to establish new therapeutic concepts for the treatment of PCa.

1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer among American men and remains the second leading cause of cancer death in 2010; approximately 217,730 men were diagnosed with PCa, and 32,050 men were expected to die from this disease in the United States [1]. Androgen and androgen receptor (AR) functions play a pivotal role in the carcinogenesis and progression of PCa, as well as in normal prostate development [2–5]. The AR is a member of the nuclear receptor (NR) superfamily as a ligand-dependent transcription factor [6]. Since cloning of the AR cDNA in 1988 [7], it has been extensively studied to elucidate how androgens activate the AR signaling pathway so as to be responsible for the progression of PCa. Huggins and Hodges in 1941 found androgen deprivation therapy (ADT) to be an effective therapy for PCa [8]. Currently, anti-androgens, in combination with surgical or medical castration, are widely used for the treatment of PCa. Both steroidal and non-steroidal anti-androgens are presently available and have shown clinical benefits as chemotherapeutic agents for PCa [9]. However, most patients relapse after an initial response to ADT, eventually developing castration resistant prostate cancer (CRPC) [10]. The possible mechanisms of CRPC development could be due to the altered sensitivity of AR to anti-androgens, mutations of AR, gene amplification of AR, changes of AR coregulators, and growth factor/kinase-activated AR activity. Therefore, it is desirable to develop potential anti-androgens or anti-AR drugs for use in PCa therapy [11].

Dihydrotestosterone (DHT) has a typical steroid structure and can bind to AR to control the development of the secondary sex

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characteristics and sex organs in males. Finasteride is a synthetic anti-androgen initially approved by the US Food and Drug Administration (FDA) in 1992 as a treatment for benign prostatic hyperplasia (BPH). It was found that the parent structure of finasteride is a 3-Oxo-4-aza-5α-androst structure modified from the original steroid structure. The structure similarity of those compounds urged us to find an anti-PCa drug or natural compound with a similar steroid structure to that of DHT or finasteride.

Salvia miltiorrhiza Bunge (Danshen) is an herb commonly used in traditional oriental medicine for the treatment of cardiovascular diseases, hepatitis, menstrual disorders, diabetes, and chronic renal failure [12,13]. More than 50 tanshinones have been isolated from Danshen. Cryptotanshinone (CTS) is one of the principal active constituents in Danshen extract, its chemical name is (R)-1,2,6,7,8,9-Hexahydro-1,6,6-trimethyl-phenanthro (1,2-b) furan-10,11-dione, and has a structure very close to DHT. In an early report [14], CTS at 7 μM was shown to inhibit the growth of DU145 PCa cells by inhibiting the STAT3 signal pathway. Due to the lack of STAT3 activation in LNCaP and PC-3 cells, the earlier report also concluded that CTS does not effectively inhibit the growth of those two cells. However, this earlier report never focused on testing the CTS effects on androgen-stimulated AR biological events and androgen/AR regulated cancer cell growth. To date, CTS was also reported to show a variety of biological activities, such as anti-angiogenic [15], antioxidant [16], anti-inflammatory [17], and anti-human hepatocellular carcinoma effects [18]. In addition, CTS was reported to decrease 17α-hydroxy progesterone and reduce androgen synthesis [19]. However, there is no related report about the effect of CTS on AR inhibition.

In this study, we are the first group to discover the ability of CTS to regulate AR transactivation. We also analyzed CTS's inhibitory effects on mRNA expressions of AR target genes that are DHT-mediated in different AR positive cells. To test the functional activity of AR, we further examined the protein levels of prostate-specific antigen (PSA), as the PSA is an AR target gene. We used AR positive PCa cell lines (LNCaP and CWR22Rv1), AR negative PCa cell line (PC-3), and non-malignant prostate epithelial cell line (RWPE-1) as model systems to investigate the differential cell growth inhibition effects of CTS. Our data showed that low concentrations of CTS could inhibit the growth of LNCaP and CWR22Rv1 cells, but had little effect on AR negative PC-3 cells and non-malignant prostate epithelial RWPE-1 cells. Together, our data showed CTS could effectively inhibit AR activity via inhibiting the AR dimerization and AR-coregulator complex formation. Therefore, CTS is a potential anti-AR compound for the therapeutic treatment for PCa.

2. Materials and methods

2.1. Reagents

Commercial compounds and reagents include DHT, dexamethasone (DEX), RU486, progesterone, hydroxflutamide (HF), 17β-estradiol (E2) and ICI 182,780 (ICI) [Sigma, St Louis, MO], ethanol (EtOH), ethyl acetate (EtOAc), petroleum ether, methanol, and chloroform ($CHCl_3$) [Sinopharm Chemical Reagent Co., Ltd. (SCRC), Shanghai]. All other chemicals and solvents used in this study were of reagent grade or high performance liquid chromatography (HPLC) grade.

2.2. Plant extracts preparation

Fresh whole plants of Danshen were purchased from a Chinese medicinal herb market in Jiangsu. Whole air-dried roots of Danshen (100 g) were extracted with 95% EtOH at effluent temperature for 2 h twice. The solvent was evaporated to obtain crude extract (9.31 g), which was applied to the silica gel column chromatography, eluted by n-Hexane–EtOAc mixture and petroleum ether-EtOAc ether as mobile phases to obtain different fractions, based on the TLC pattern. Cryptotanshinone (16 mg) was separated and purified under silica gel column chromatography with a solvent system of petroleum-EtOAc mixture, and identified by comparison of NMR and MS spectral data with reference values [20,21].

2.3. Cell culture

Cells were cultured at 5% CO_2 and 37 °C. LNCaP is an androgen-responsive and androgen dependent-human PCa cell line with a mutant AR (T877A); CWR22Rv1 is an androgen-responsive but androgen-independent human PCa cell line, which expresses endogenous AR; DU145 and PC-3 are androgen-independent human PCa cell lines that lack expression of AR. LNCaP, CWR22Rv1, DU145, and PC-3 cell lines were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin).

RWPE-1, the non-malignant human prostate cell line was maintained in keratinocyte serum-free medium (Invitrogen, catalog No. 10724) and supplements (Invitrogen, catalog No. 37000-015).

HEK 293 cell line was generated by transformation of human embryonic kidney cell cultures, and maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% FBS and antibiotics.

2.4. Plasmids

The plasmids used were pSG5AR, full-length cDNA of wild-type human AR; MMTV-Luc (MMTV) a luciferase reporter plasmid; and plasmids pSG5 progesterone receptor (pSG5PR), pSG5 glucocorticoid receptor (pSG5 GR), PIRES-flag-ARt877a, pSG5, pSG5AR(N-DBD), pcDNA3.1-ERα, pGL3 $(ERE)_3$-Luc, pRL-TK pCMX-VP16-ARA70, Gal4-AR-LBD, and Gal4-RE-Luc were constructed as previously described [22–25].

2.5. Luciferase assays

Luciferase activity, transfections, and reporter gene assays, were performed using Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol. HEK 293 cells, lacking functional AR or ERα, were transfected with wild-type AR or ERα expression plasmid and reporter gene. Briefly, 2×10^4 HEK 293 cells were plated on 24-well dishes with 10% charcoal stripped (CS)-FBS DMEM medium for 24 h, medium was refreshed, and each well of cells were transfected with 0.6 μg of pSG5-AR, pSG5-PR, pSG5-GR (for AR, PR, or GR transfections, respectively), 0.3 μg MMTV-Luc, and 1 ng pRL-TK-Luc, or with 0.3 μg cDNA3.1-ERα, pGL3 $(ERE)_3$-Luc, and 1 ng pRL-TK-Luc for ERα transfections for 24 h. After transfection, the medium was refreshed to 10% CS-FBS medium and cells treated with various concentrations of CTS in the presence or absence of 1 nM DHT and/or 5 μM HF for 24 h for AR transfections, and treated with serial concentrations of CTS or 10 μM anti-estrogen (ICI 182,780) in the absence or presence of 10 nM E2 for 24 h for ER transfections. For the PR and GR reporter activity assay, 10 nM progesterone, or DEX were added, respectively. To inhibit the progesterone-PR or DEX-GR activities, 10 μM RU486 was added.

Briefly, 5×10^4 LNCaP cells or CWR22Rv1 cells were plated on 24-well dishes with 10% CS-FBS RPMI-1640 medium for 24 h, medium was refreshed and cells transfected with 0.3 μg MMTV-ARE-Luc and 1 ng pRL-TK-Luc for 24 h. After transfection, the medium was changed to 10% CS-FBS medium for treatment with various concentrations of CTS in the presence or absence of 1 nM DHT and/or 5 μM HF for 24 h. These cells were then harvested and assayed for luciferase activity using the Dual Luciferase Assay System. Data were expressed as relative luciferase activity normalized to the internal Renilla luciferase control.

For the mammalian 2-hybrid assay to determine the AR N–C interaction and AR–AR coregulator interaction, HEK 293 cells were plated on 24-well dishes with 10% CS-FBS DMEM medium for 24 h. Cells were transfected with pGal4-RE-Luc reporter plasmid, pGal4-ARDBD-LBD (AR DNA binding domain and ligand binding domain), pCMX-VP16-AR or pCMX-VP16-ARA70 plasmids as indicated in the figure legends. After 24 h transfection, the medium was refreshed to 10% CS-FBS medium and cells were treated with 1 nM DHT and/or CTS for an additional 24 h. tk-RL luciferase was co-transfected as the internal control. Cells were then harvested for the dual luciferase assay (Promega, WI).

2.6. RT-PCR and real-time PCR

Total RNA was extracted from PCa cells using Trizol (Invitrogen). Reverse transcription was performed using the Superscript first-stand synthesis kit (Invitrogen). Quantitative real-time PCR analyses using the comparative CT method were performed on an ABI PRISM 7700 Sequence Detector System using the SYBR Green PCR Master Mix kit (Perkin Elmer, Applied Biosystems, Wellesley, MA, USA) according to the manufacturer's instructions. After an initial incubation at 50 °C for 2 min and 10 min at 95 °C, amplification was performed for 40 cycles at 95 °C for 20 s, 65 °C for 20 s, and 72 °C for 30 s. Specific primer pairs were determined with the Primer-Express program (Applied Biosystems). The PSA primer pairs were 5′-AGG CCT TCC CTG TAC ACC AA-3′ and 5′-GTC TTG GCC TGG TCA TTT CC-3′. The TMPRSS2 primer pairs were 5′-GTA CAC TGT TTC CAT GTT ATG-3′ and 5′-AAT AAG AAG GAG TCA TTT GAG-3′. The TMEPA1 primer pairs were 5′-CCT TCT CTT CCC CTT TCC ATC TCC-3′ and 5′-GTC CCG CCA ACC CCA AAT CTA TCT-3′. The normalization control used was β-actin, and the primers used were 5′-TCA CCC ACA CTG TGC CCC ATC TAC GA-3′ and 5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′.