Tanshinone IIA Inhibits the Growth of LNCaP Cells by Blocking the Transcriptional Activity of Androgen Receptor

Bei-Yu Wu¹, #, Chun-Ting Liu¹, #, Mao-Feng Sun², ³, Hong-Yo Kang⁴, ⁵, Ching-Yuan Wu⁴, ⁶, *

¹Department of Chinese Medicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan
²Department of Acupuncture, China Medical University Hospital, Taichung, Taiwan
³School of Chinese Medicine, China Medical University, Taichung, Taiwan
⁴Graduate Institute of Clinical Medical Sciences, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan
⁵Center for Menopause and Reproductive Research, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan
⁶Department of Chinese Medicine, Chiayi Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Chiayi, Taiwan

(Received 17th April 2014, accepted 6th June 2014)

Androgen receptor (AR) is a nuclear transcription factor of the hormone receptor family. For some prostate cancers, named AR-dependent prostate cancer, it plays an important role for the development and progress of prostate cancer. In this study, we showed 0.1μM tanshinone IIA can inhibit the expression of mRNA and protein level in AR downstream proteins. It suggests tanshinone IIA can block the transcriptional activity of activated ARs by lower concentration. We also showed tanshinone IIA can suppress stability of AR protein, but not the mRNA level of AR. We also found tanshinone IIA can inhibit the growth of LNCaP cells, AR-dependent prostate cancer cell line. This inhibitory effect is stronger on LNCaP cells stimulated by dihydrotestosterone (DHT) than without DHT, suggesting that tanshinone IIA can inhibit the growth of LNCaP cells through affecting the function of AR. In summary, we discovered tanshinone IIA can efficiently inhibit AR transactivation, block AR target genes expression in low concentration (0.1 μM), and then inhibit cell growth in AR positive LNCaP cells. Our results suggest that tanshinone IIA could be a potential agent for the treatment of prostate cancers.

Key words: Tanshinone IIA, androgen receptor, prostate cancer, LNCaP cells

¹Bei-Yu Wu and Chun-Ting Liu contributed equally to this work.

*Correspondence to: Ching Yuan Wu, Department of Chinese Medicine, Chiayi Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No. 6, W. Sec., Jiapu Rd., Puzi City, Chiayi County 613, Taiwan (R.O.C.), Tel: +886-5-3621000, Fax: +886-5-3623002, E-mail: smbepigwu77@gmail.com
Introduction

Prostate cancer is the most common malignant disease as well as the second leading cause of death of male cancer patients in the US. Since prostate cancer is responsive for androgen suppression, androgen deprivation therapy (ADT) is mainly used after medical or surgical castration. However, poor response to secondary hormonal manipulations has been observed in most patients after an initial response to ADT\textsuperscript{1,2}. Prostate cancer that progress with castrate levels of testosterone is classified as “castration-resistant prostate cancer (CRPC)\textsuperscript{3}”. The development of hormonal resistance is the major cause of this short period of responsiveness. To improve clinical outcome, it is important to develop new drugs which can more effectively inhibit AR signaling.

Androgen receptor (AR) is a member of the steroid nuclear receptor superfamily. AR is a nuclear transcription factor activated by binding of AR ligands which then enables the regulation of expressions of target genes including prostate-specific antigen (PSA) and transmembrane protease, serine 2 (TMPRSS2)\textsuperscript{4,5}. Dihydrotestosterone (DHT) is a sex steroid and AR ligand. It can activate androgen receptor and induce the transcription of androgen receptor target genes. It also can promote the proliferation of androgen receptor-positive prostate cancer cell lines, like LNCaP cell. The initiation and progression of prostate cancer requires the completeness and functioning of AR signaling despite castration levels of testosterone\textsuperscript{1,4}. AR is also expressed and is necessary for the growth of CRPC cells although androgen is dispensable for CRPC survival. The dependence of CRPC on AR may boil down to several mechanisms, including point mutation in AR, amplification of AR, and changes in expression of AR co-regulatory proteins\textsuperscript{6-8}. These alterations will lead the native AR to develop into “super AR” which represents the species responding to reduced concentrations of androgens or to other ligands. Therefore, the inhibition of AR function becomes an imperative goal in the search of new compounds and the development of novel anti-prostate cancer drugs.

Dried root of \textit{Salvia miltiorrhiza} Bunge is used to treat a number of cardiovascular and endocrine diseases, including coronary artery disease, angina pectoris, hepatitis and menstrual disorder in traditional Chinese medicine\textsuperscript{9}. Tanshinone IIA (Fig.1), extracted from the dried root of \textit{S. miltiorrhiza} Bunge, belongs to the group which contains abietane type-diterpene quinone\textsuperscript{10}. While cryptotanshinone was previously shown to possess the powerful antibacterial activity among the tanshinones and inhibit the growth of the androgen-independent prostate cancer cell line in vitro and in mice\textsuperscript{11,12}, the effects of tanshinone IIA and its mechanism of action on androgen-dependent prostate cancer cells remains unclear. In our previous study\textsuperscript{12}, we found cryptotanshinone, which also belong to abietane type-diterpene quinone pigments, can inhibit the growth of AR-positive prostate cancer

Tanshinone IIA

![Structure of tanshinone IIA.](image-url)
cells via blocking AR signaling pathway and inhibit the transcriptional activity of AR-target genes. Because tanshinone IIA has the similar structure with cryptotanshinone, we intend to investigate its function in prostate cancer cell proliferation, followed by the effect of tanshinone IIA on the transcriptional activity of AR to decipher the molecular mechanism.

Materials and Methods

Cell Culture and Treatment

The human prostate cancer cell lines (LNCaP cells and PC3) were obtained from the American Type Culture Collection. Human prostate cancer PC3 cells were maintained in Dulbecco’s Minimum Essential Medium (DMEM) (Invitrogen Corp., Carlsbad, CA) containing penicillin (25U/ml), streptomycin (25μg/ml) and 10% fetal bovine serum (FBS). The LNCaP were cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA), supplemented with 10% FBS at 37 °C and 5% CO₂. Stable clones of PC3-AR cell line were obtained after puromycin selection as previous described. Tanshinone IIA was obtained from Sigma, US (T4952, the purity of tanshinone IIA is >97% by HPLC and its solubility in DMSO is >5mg/mL). DHT (dihydrotestosterone) was obtained from Sigma. LNCaP cells were cultured to 60-70% confluence prior to treatment. Medium was then replaced with fresh medium containing tanshinone IIA in DMSO (dimethyl sulfoxide) at the indicated concentrations. Cells treated with DMSO alone were used as untreated controls.

XTT Assay

Human prostate cancer cell lines (LNCaP) were plated at a density of 10⁵ or 5 x 10⁵ per well, respectively, in 96-well plates, in RPMI 1640 medium containing 10% FBS. Once attached, the medium was replaced with RPMI 1640 containing 10% charcoal-dextran–treated FBS. The cells were then treated with DHT or tanshinone IIA for 24, 48, or 72 hours; and absorbance were measured using the XTT assay kit (Roche, Cat. No. 11465015001) according to the manufacturer’s instructions as described previously. The XTT formazan complex was quantitatively measured at 492 nm using an ELISA reader (Bio-Rad Laboratories, Inc.).

Luciferase Assay

LNCaP were transiently transfected with the androgen response element (ARE)-luciferase construct or prostate specific antigen (PSA)-luciferase construct (both constructs are kindly gifts from Dr. Hong-Yo, Kang). PC3-AR cells were transiently transfected with the mouse mammary tumor virus (MMTV)-luciferase construct using the Superfect reagent (Qiagen, Cat. No. 301307) according to the manufacturer’s instructions. Cells were cotransfected with the Reni-luciferase (SV40) plasmid as the internal control. The cells were treated with the indicated drugs or left untreated for 48 hours posttransfection. Cells were harvested and luciferase activity was measured using the Dual Luciferase Assay Kit (Promega, Cat. No. E1980) according to the manufacturer’s instructions.

Quantitative Real Time PCR

Total RNA was extracted from LNCaP cells using the TRIzol reagent (Invitrogen, Cat. No. 15596-026) according to the manufacturer’s instructions. Reverse transcription was performed using the Superscript first strand synthesis kit (Invitrogen, Number: 11904018). 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. Following 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 sec, 65°C for 20 sec and 72°C for 30 sec. Specific primer pairs were determined with the PrimerExpress program (Applied Biosystems). The primer sequences were 5’-GCCTTGCTCTCTAGCCTCAA-3’ and 5’-GGTCGTCCACGTGTAAGTTG-3’.

Western Blot Analysis

Western blot analyses were performed as described previously12. For Western’s blotting, cellular extracts of LNCaP cells treated with or without DHT and TIIA for 1 hour were prepared according to the manufacturer’s instructions. The equal amounts of protein were fractionated on a 7.5 or 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% nonfat dried milk for 30 minutes and incubated in primary antibody for 3 hours in room temperature. The primary antibodies used were: AR (Santa Cruz, N-20, sc-816, ratio: 1:1000), PSA (Santa Cruz, C-19, sc-7638, ratio: 1:1000), anti-α-tubulin antibody (IB: 1:10000, Sigma). The primary antibody and secondary antibody were diluted with 1% nonfat dried milk in 1X TBST (Tris-Buffered Saline Tween-20). Blots were washed by 1X TBST and incubated in horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (Santa Cruz, ratio: 1:5000) for one hour in room temperature. After washing by 1X TBST again, protein signal was detected by chemiluminescence, using the Super Signal substrate (Pierce, Number: 34087).

Statistical Analyses

All values were the means ± standard deviations (SD) of replicate samples (n=3 to 6, depending on the experiment) and experiments were repeated a minimum of three times. Differences between two groups were assessed using the unpaired two-tailed Student’s t-test or by ANOVA if more than two groups were analyzed. The Tukey test was used as a post-hoc test in ANOVA for testing the significance of pairwise group comparisons. P-values <0.05 were considered statistically significant in all comparisons. SPSS version 13.0 for windows (LEAD technologies, Inc.) was used for all calculations.

Results

Human prostate cancer cell line (AR-positive LNCaP cells), which is responsive to androgen stimulation15, was used as our model to study the effect of tanshinone IIA. Since 10 μM or higher concentration of tanshinone IIA has been shown to have better inhibitory effect on prostate cancer cell growth11, we used the concentration of 10 μM to perform experiments. Our results showed that the low dose (10 μM) of tanshinone IIA could significantly inhibit the proliferation of LNCaP cells without DHT in a time-dependent manner and, more importantly, the growth of AR-positive prostate cancer cells (Fig. 2). Secondly, this data suggests that tanshinone IIA may decrease AR-dependent prostate cancer cell growth.

The transcriptional activity of activated AR plays an important role in keeping the growth of AR-dependent prostate cancer cells (LNCaP cells). In our results, tanshinone IIA can inhibit the growth of AR-positive prostate cancer cells (Fig. 2). Secondly,
Bei-Yu Wu, Chun-Ting Liu, Mao-Feng Sun, Hong-Yo Kang, Ching-Yuan Wu

we investigated the effect of tanshinone IIA on the transcriptional activity of AR by luciferase reporter gene assay. Prostate specific antigen (PSA)-luciferase construct represents the AR target gene, PSA. Androgen response element (ARE)-luciferase construct contains androgen receptor binding sites (androgen response element) in the plasmid. The activated androgen receptors can bind to the androgen response elements and induce the expression of luciferase. We used LNCaP cells transiently transfected with ARE-luciferase or PSA-luciferase construct and PC3-AR cells transfected with the mouse mammary tumor virus (MMTV)-luciferase construct as reporter plasmids. We also used Reni-luciferase (SV40) as internal control plasmid for both. After treated with or without various doses of tanshinone IIA and 10 nM DHT for 24 hours, the activity level of the luciferase of PSA, ARE and MMTV were decreased both in LNCaP cells (Fig. 3 A, B) and PC3-AR cells (Fig. 3 C) by 0.1 µM of tanshinone IIA. This data suggests that low

Fig. 2  LNCaP cell proliferation is inhibited by tanshinone IIA. The growth rates of LNCaP cell line as measured by XTT assay after 1-3 days of culturing in the presence of 10 µM of tanshinone IIA (TIIA) with or without 10 nM DHT treatment. There were three replicates for each experiment and the results represent the mean± S.D. of five experiments. Asterisks (*) mark samples significantly different from mock treatment with $P<0.05$.

Fig. 3  Transcriptional activity of AR is inhibited by tanshinone IIA. LNCaP cells (A, B) were transiently transfected with the androgen response element (ARE)-luciferase construct or the prostate specific antigen (PSA)-luciferase construct. PC3-AR cells (C)
were transiently transfected with the mouse mammary tumor virus (MMTV)-luciferase construct. Renilla-luciferase (SV40) was used to control transfection efficiency in both cell types. Varying concentrations of tanshinone IIA were added in the presence or absence of 10nM DHT and transcriptional function of AR was then quantified by the dual-luciferase assay after 48 hours. Each experiment was performed in triplicate and the results represent the mean± S.D. Asterisks (*) mark samples significantly different from tanshinone IIA treatment with P <0.05. DHT: 10 nM of dihydrotestosterone. TIIA: 0.1, 1 or 10 µM tanshinone IIA.

Based on Figure 3, we observed that tanshinone IIA could inhibit PSA-luciferase, MMTV-luciferase and ARE-luciferase activity in a dose-dependent manner. To further confirm the effect of tanshinone IIA on the AR downstream target genes, we checked AR target genes in LNCaP cells, AR-positive cells by q-PCR. As shown in Figure 4, LNCaP cells can promote the mRNA levels of AR-target genes, including prostate-specific antigen (PSA) and transmembrane protease, serine 2 (TMPRSS2), with treatment of DHT. After treatment of tanshinone IIA, our data showed that PSA and TMPRSS2 mRNA levels were effectively suppressed in a dose-dependent manner in LNCaP cells (Fig. 4). Additionally, we found that tanshinone IIA can inhibit the transcriptional activity of ARs in LNCaP cells, but the mechanism is still unclear. To assess whether tanshinone IIA can affect the expression of AR protein, LNCaP cells were cultured in 10% CD-FBS for 2 days and then treated with indicated drugs for 48 hours. After harvesting the cell lysate and Western’s blotting assay, our data displayed that the protein levels of AR were stabilized by DHT and approximate 1-10 µM tanshinone IIA can reduce AR protein stability in LNCaP cells (Fig. 5). We also confirmed the effect of tanshinone IIA on DHT-induced protein levels of the AR downstream target gene, PSA, in LNCaP cells. As consistent with Figure 4, our data showed that the protein levels of PSA were induced by DHT, and 0.1 µM tanshinone IIA can significantly inhibit the PSA protein expressions (Fig. 5). After collecting the
total mRNA and qPCR, we showed that there was no significant difference in AR mRNA levels between untreated and treated cells after the treatment with different concentrations of tanshinone IIA in LNCaP cells (Fig. 6). Our data suggests that tanshinone IIA can inhibit AR function by suppressing AR protein stability, but not affect the AR mRNA level.

**Discussion**

AR-mediated signaling pathways play a critical role in the growth and survival of primary prostate cancer cells. In previous studies, the antitumor activity of tanshinone IIA has been well studied in several types of cancers including prostate cancer. However, its exact mechanism of action in AR-positive prostate cancer cells is not completely understood. Our current study demonstrated that tanshinone IIA inhibited the proliferation of LNCaP cells, an androgen-responsive and androgen-dependent human prostate adenocarcinoma cells. Then, the suppression of tanshinone IIA on LNCaP cells may be related to AR function. In our data, tanshinone IIA can both inhibit the luciferase activity of PSA and ARE in LNCaP cells, and MMTV in PC3-AR cells. In consistent result, tanshinone IIA also can inhibit the PSA protein level and the PSA, TMPRSS2 mRNA level, the AR-target genes. Moreover, we also found the stability of AR protein was reduced by tanshinone IIA treatment, but the AR mRNA level was not affected. This data suggests tanshinone IIA can block the transcription activity of AR and then inhibit the growth of LNCaP cells, AR-positive prostate cancer cells by blocking the function of AR protein.
In our previous study\textsuperscript{12}, we found cryptotanshinone can profoundly inhibit the AR downstream genes in the concentration of 1 µM (Luciferase assay and q-PCR) and the medium lethal dose (LD\textsubscript{50}) of cryptotanshinone was 20 µM for LNCaP cells. In the current study, our data showed tanshinone IIA can dramatically inhibit the AR target genes in lower concentration (0.1 µM) and LD\textsubscript{50} was 10 µM for LNCaP cells. It suggests tanshinone IIA has a better specificity targeting the AR signaling and stronger inhibiting the AR-positive tumor cells. This finding was consistent with an earlier study, in which IC\textsubscript{50} of cryptotanshinone and tanshinone IIA for different prostate cancer cell lines were approximately 10-25 µM and 8-15 µM, respectively\textsuperscript{11, 19-22}. Interestingly, we found as low as 0.1µM tanshinone IIA could decrease DHT-induced transcriptional activity of activated AR. This finding about distinct concentration may provide clues for understanding the different mechanism of tanshinone IIA inhibiting prostate cancer cell growth.

Since AR is essential for the development of castration-resistant prostate cancer, targeting of AR signaling becomes a valid therapeutic strategy in recent decades. These investigational focuses could be summarized to androgen/androgen receptor axis, including AR expression, AR-driven transcription, AR splice variant, androgen synthesis/transport, and AR interactions with oncogenic signaling pathway\textsuperscript{23}. Combining out data with previous studies, the target points of tanshinone IIA on prostate cancer cells may be this androgen/androgen receptor axis and subsequent cell cycle/apoptosis regulation. The mechanism should be investigated more in-depth to identify the precise action point of tanshinone IIA for future therapeutic application.

Conclusions

In conclusion, the data from this study indicated that tanshinone IIA can decrease the proliferation of AR positive LNCaP cells through inhibiting the transcriptional activity of AR target genes and by suppressing the level of AR mRNA and the stability of AR protein. These results collectively suggest that tanshinone IIA could be a potent anticancer agent for castration resistant prostate cancer.

Acknowledgements

This work was supported by grant CMRPG6C0261 from Chang Gung Memorial Hospital, and NSC101-2320-B-182A-005 and NSC 102-2320-B-182A-007 from the National Science Council to Dr. Ching Yuan Wu.

Conflict of Interest

The authors declare no conflict of interest.

References

3. Scher HI, Halabi S, Tannock I, Morris M, Sternberg CN, Carducci MA, Eisenberger MA, Higano C,
Bei-Yu Wu, Chun-Ting Liu, Mao-Feng Sun, Hong-Yo Kang, Ching-Yuan Wu


丹參酮 IIＡ 藉由阻斷雄性素受體的轉錄活性而抑制 LNCaP 細胞的生長

吳蓓禹 1, 2*, 劉俊廷 1, 2*, 孫茂峰 3, 4, 康宏佑 4, 5, 吳清源 4, 6*

1. 高雄長庚紀念醫院中醫科系，高雄，台灣
2. 中國醫藥大學附設醫院針灸科，台中，台灣
3. 中國醫藥大學中醫學院，台中，台灣
4. 長庚大學臨床醫學研究所，高雄，台灣
5. 高雄長庚紀念醫院更年期及生殖醫學研究中心實驗室，高雄，台灣
6. 嘉義長庚紀念醫院中醫科，嘉義，台灣

(103 年 4 月 17 日受理，103 年 6 月 6 日接受刊載)

雄性素受體（Androgen receptor, AR）是激素受體家族的一種核轉錄因子。在雄性素受體依賴性前列腺癌（AR-dependent prostate cancer）中，雄性素受體對於前列腺癌的發生與進展扮演著重要的角色。我們的研究發現，0.1 μM 的 tanshinone IIＡ 即能抑制雄性素受體下游蛋白的 mRNA 和蛋白質表現的水平，顯示 tanshinone IIＡ 在低濃度即能阻斷活性的雄性素受體的轉錄活性。此外，我們亦發現，tanshinone IIＡ 能抑制雄性素受體的穩定性，但不影響雄性素受體的 mRNA 水平。同時我們發現，丹參酮 IIＡ（tanshinone IIＡ）能抑制雄性素受體依賴性前列腺癌細胞株 LNCaP 細胞的生長。此作用在加入雙氫睾酮（dihydrotestosterone，DHT）活化之 LNCaP 細胞表現的較沒有加入 DHT 之 LNCaP 細胞更為明顯，表示 tanshinone IIＡ 可能透過影響雄性素受體的功能來抑制 LNCaP 細胞的生長。綜合上述，我們發現 tanshinone IIＡ 在低濃度能有效的抑制雄性素受體的活性、阻斷雄性素受體目標基因（target gene）的表現，進而抑制前列腺癌細胞株 LNCaP 細胞的生長。我們的研究認為，tanshinone IIＡ 有潛力成為治療前列腺癌的藥物。

關鍵字：丹參酮 IIＡ，雄性素受體，前列腺癌，LNCaP 細胞

* 共同第一作者
* 聯絡人：吳清源，嘉義長庚紀念醫院中醫科，613 台灣嘉義縣朴子市嘉朴路西段 6 號，電話：05-3621000，傳真：05-3623002，電子郵件信箱：smhepigwu77@gmail.com