The Anticancer Effect of Theranekron® on Androgen-Dependent Prostate Cancer Cells

Running title: Theranekron®: Therapeutic candidate for Prostate carcinogenesis

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ABSTRACT

Objectives
Prostate cancer (PCa) is a significant health problem in men worldwide. Although there are numerous treatment choices for PCa, acquired resistance limits the success of treatment. Therefore, there is a need for new approaches as a powerful resource for use in an alternative or supportive therapeutic strategy for anti-cancer therapeutics. Theranekron® is a commercially available alcoholic extract of Tarantula cubensis. Recent studies showed the potent anticancer effect of theranekron in human tumors, including PCa. Herein, we comparatively examined the anti-proliferative activity of theranekron and biochemical action on androgenic signal and cell cycle-related cyclin proteins in androgen-dependent PCa cells, LNCaP, VCaP and 22Rv1.

Materials and Methods
Human androgen-dependent PCa cells, LNCaP (CRL-1740™), 22Rv1 (CRL-2505™) and VCaP (CRL-2876™) were used to evaluate the effect of theranekron in vitro. The impact of theranekron on cell viability was evaluated by WST-1 based viability test. Its impact on AR, cyclin A2, cyclin B1 and cyclin E1 was examined by immunoblotting studies. To test the anti-malignant effect of theranekron on 3D tumor formation of PCa cells was tested by soft agar assay.

Results
Our results indicated that theranekron treatment significantly reduced the viability of PCa cells. It remarkably decreased the protein levels of AR, cyclin A2, cyclin B1 and cyclin E1 in a dose-dependent manner. Additionally, theranekron administration strongly limited the 3D tumor formation of LNCaP, 22Rv1 and VCaP cells.

Discussion and Conclusion
Our findings strongly suggest that theranekron may offer potent therapeutic efficacy against androgen-dependent PCa cells. Moreover, it may be a potent component for preventing the acquired resistance to chemotherapeutics.

Keywords: Androgen receptor, Anticancer, Cell-cycle, Theranekron, Prostate cancer, Venom

INTRODUCTION
Cancer affected 17 million people in 2018 and led to the death of 9.6 million people worldwide¹. Prostate cancer (PCa) is a significant health problem and the second most common cancer type in men worldwide. Surgical intervention, cryotherapy, chemotherapy and androgen deprivation therapy are frequently preferred for the treatment of PCa²,³. However, both resistance to chemotherapeutics and conversion of PCa to castration resistance prostate cancer (CRPC) type substantially limit the success of treatment⁴.

Animal venoms have a complex rich mixture of various bioactive molecules and thereby can exhibit numerous pharmacological actions in the cells. In recent years, it has been recommended as a potent resource for use in an alternative or supportive therapeutic strategy for anti-cancer therapeutics and its biochemical activities are being studied on a large scale by numerous research groups⁵,⁶.

* Tarantula cubensis, also known as the Cuban tarantula, is a large arachnid in the family Theraphosidae⁷.

* Theranekron® is a commercially available alcoholic extract of *Tarantula cubensis* and it widely uses in veterinary for the treatment of numerous animal diseases such as panaritium, laminitis, foot rot, arthritis,
abscesses, and several injuries\(^7,8\). Various studies have demonstrated that theranekron exerts various biochemical actions in mammalian human cells, including anti-inflammatory, wound healing and anticancer as well\(^9,10\). Moreover, it has been reported that resorptive, regenerative, antiphlogistic and demarcative effects in proliferative and necrotic tissues\(^1,12\). The antitumor properties of theranekron have been reported on canine mammary tumors and also in vitro human cancer models\(^13,14\). Very recent in vitro studies have been focused on the effects of theranekron on different cancer models, including breast, lung, osteosarcoma and prostate\(^15\). Our previous study showed that androgen-sensitive human prostate adenocarcinoma cell LNCaP was more sensitive to Theranekron than the normal prostate cell line, PNT1A\(^13\). On this basis in the present study, we investigated the effects of theranekron on LNCaP, VCaP and 22Rv1 cells, which are known as androgen-dependent PCA cell lines.

Herein, we comparatively examined the anti-proliferative activity of theranekron and investigated the biochemical action on androgenic signal and cell cycle-related cyclin proteins by immunoblotting. Also, we tested the action of theranekron on anchorage-independent cell growth of androgen-dependent PCA cells by using a 3D cell culture model. Our findings suggest that theranekron may offer potent therapeutic efficacy against androgen-dependent PCA cells. Moreover, it may be a potent component for preventing the acquired resistance to chemotherapeutics.

**MATERIALS AND METHODS**

**Materials**

Cell culture supplements such as fetal bovine serum (FBS), L-glutamine, Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 were obtained from Capricorn-Scientific. Theranekron\(^\text{TM}\) was provide from Richter- Pharma AG, Wels, Austria. Rabbit polyclonal anti-cyclin A2 (#201500)(1:2000), anti-cyclin B1(#12231)(1:2000) and anti-cyclin E1 (#208080)(1:2000) were purchased from Cell Signaling Technology. Polyclonal rabbit antibody anti-AR (#22089-1-AP)(1:2500) was obtained from Proteintech. Mouse monoclonal anti-beta-actin (#A5316)(1:10000) antibody was provided from Sigma Aldrich. HRP-conjugated goat anti-rabbit (#31460)(1:5000) and anti-mouse (#31430)(1:5000) IgG (H+L) were obtained from Thermo Scientific.

**Cell culture**

Human androgen-sensitive prostate adenocarcinoma cell line, LNCaP (CRL-1740\(^\text{TM}\)) and 22Rv1 (CRL-2505\(^\text{TM}\)) and VCaP (CRL-2876\(^\text{TM}\)) were obtained from American Type Tissue Culture (ATCC). LNCaP and 22Rv1 cells were cultured in RPMI 1640. VCaP cells were propagated in DMEM. All cell culture medias were enriched with 10% FBS, 2 mM L-glutamine and 5 mg ml\(^{-1}\) penicillin/streptomycin (Capricorn-Scientific). Cultured cells were kept in a humidified atmosphere of 5% CO\(_2\) and 95% air at a constant temperature of 37 °C.

**Cell viability assay**

Cells were seeded into a 96-well plate (10000 cells/well) and grown for 24 hours. Then cells were treated with theranekron in various doses for 48 hours. WST-1 cell viability assay (Takara) was performed according to the manufacturer's instructions. The absorbance was determined at 450nm with 600nm set as the reference wavelength by microplate spectrophotometer (BioTek, Epoch 2). Averages of the absorbance values were calculated and viability rates were presented in the graph as a % fold change. IC\(_{50}\) values of theranekron were determined by GraphPad Prism 5 software.

**Western blotting**

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and then centrifuged at 14,000 rpm for 20 min at 4°C. Insoluble phase was removed and supernatant was collected. The concentration of total protein was determined by bicinchoninic acid (BCA) assay (Takara). 30 μg of protein was used in immunoblotting studies. Samples were denatured in 4x Laemml buffer at 70°C for 15 min and were separated on hand-cast polyacrylamide gels. Separated proteins were transferred to a Immobilon®-P polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in 5% nonfat dry milk in Phosphate-buffered saline (PBS) containing 0.1% Tween (TBS-Tween) for 1 hour at room temperature and then primary and secondary antibodies were applied for 2 hours at room temperature, respectively. Target proteins were monitored by using enhanced Clarity™ chemiluminescence (ECL) solution (Bio-Rad) in ChemiDoc XRS+ (Bio-Rad). The densitometry of protein bands was calculated by Image Studio™ Lite (LI-COR®).

**Soft agar assay**

Soft agar colony formation assay was adapted according to Borowic\(^1\). Equal volumes of 2x DMEM and 2x RPMI-1640 with 20% FBS and sterile 1.2% low-melting agar were gently mixed and added to 12 well cell culture plate. Cell suspensions prepared in 150 μl media were mixed with 250 μl of 2x DMEM or 2x RPMI 1640 containing 20% FBS and 250 μl of 0.6% agar and then transferred to the top of solidified bottom agar layer. Theranekron was applied to the cells, and the images of growing colonies were taken by Sunny SopTop inverted microscope and OD400UHW camera system. Colonial growth was quantified by taking from independent areas to 5 images and counting.

**Statistical Analysis**
RESULTS
Theranekron decreases the viability of human androgen-dependent PCA cells.
To investigate the effect of theranekron on viability of androgen-dependent LNCaP, 22Rv1 and VCaP PCA cells, we performed the WST-1 based cell viability test. Theranekron doses used in the study were selected according to the results of our previous study with LNCaP cells. For this aim, we treated the PCA cells with 12.5 µg/ml and 25 µg/ml doses of theranekron for 24 hours and then cell viability was measured. Our findings revealed that theranekron administration significantly decreased the cell viability of all tested PCA cells in a dose-dependent manner (Figure 1a, b). Besides, we determined that VCaP cell was more susceptible to the theranekron than LNCaP and 22Rv1 cells.

Theranekron decreases AR levels and induces cell cycle arresting in PCa cells.
To evaluate the action of theranekron on cell cycle-related proteins of androgen-dependent PCa cells, we treated the LNCaP, VCaP and 22Rv1 cells with various doses of theranekron and then protein expression levels of cyclin A2, cyclin B1 and cyclin E1 were analyzed by immunoblotting studies. We found that theranekron application decreased the expression level of all tested cyclin proteins in a dose-dependent manner (Figure 2a, b). Also, we tested androgen receptor (AR) protein levels, our data indicated that theranekron administration remarkably reduced the expression level of AR proteins in all androgen-dependent PCA cells (Figure 2a, b). In these studies, beta-actin expression was used as a loading control.

Theranekron remarkably reduces the 3D tumor formation of PCa cells.
Most animal model trials fail because bioactivity tests performed in monolayer culture systems are insufficient to mimic the real tumor formation and tumor environment. Therefore, we performed anchorage-independent 3D tumor formation studies to test the effect of theranekron on PCa progression. Our data indicated that theranekron application significantly inhibited the tumor formation of LNCaP, VCaP and 22Rv1 cells and also strongly reduced the developing tumor volume in all three PCA cell lines (Figure 3a, b).

DISCUSSION
Today, a few natural compounds or their synthetic analogs are clinically used against cancer. Especially spider venoms can show potent effects on cancer cells due to their strong bioactive contents. Therefore, they are seen as potential drug candidates due to their anticancer and antinociceptive activities. Numerous spider venoms have been shown to have modes of action on cancer cells. The whole venom of Macrothele raveni triggers DNA fragmentation and activates several caspase enzymes in human breast carcinoma, cervical carcinoma and hepatocellular carcinoma cells. Lycosin-1 compound of Lycosa singoriensis venom activates mitochondrial cell death signaling in human lung adenocarcinoma, human prostate carcinoma, colon adenocarcinoma cells.

The commercially available alcoholic extract of Tarantula cubensis venom, Theranekron® is often used in veterinary medicine for the treatment of animal tumors. Therapeutically, theranekron has exhibited anticancer, anti-inflammatory, antiphlogistic, demarcative and wound healing properties in clinical studies. Also, the usage of theranekron was reported in endometritis, cutaneous papillomatosis, pododermatitis and foot and mouth lesions in veterinary medicine. Latest studies focused on the anti-cancer effect of theranekron in human cancer cells. Erzurumlu et al. reported that androgen-dependent PCa cell, LNCaP was more susceptible than androgen-independent metastatic PCA cell Du145 and healthy prostatic cell line PNT1A. Mechanistically, it affected the autophagic activity and induced endoplasmic reticulum (ER) stress in androgen-dependent PCA cells. Also, it markedly reduced the epithelial-mesenchymal transition (EMT) of LNCaP cells.

Herein, we focused on the impact of theranekron in androgen dependent PCA cells, we comparatively investigated the roles on androgenic signal, cell cycle and therapeutic impact on the 3D tumor formation of PCA cells. Firstly, we examined the effects of theranekron on cell viability in LNCaP, VCaP and 22Rv1 cells. Our findings indicated that theranekron administration more strongly decreased the viability of VCaP cells compared to LNCaP and 22Rv1 cells (Figure 1). Also, theranekron doses in all applications effectively decreased the viability in all tested cell lines in a dose-dependent manner (Figure 1) (Table 1).

The androgenic signal is a crucial mechanism in the progression of PCA cells. AR protein is induced by androgens in androgen-dependent PCA cells and then expression of AR target genes is stimulated through the specialized transcriptional program. AR target genes include proto-oncogenic gene products that support prostate tumorigenesis. Therefore, suppression of AR signaling is among the major approaches in the therapeutic choices developed for PCA. We evaluated the effect of theranekron on AR protein levels in LNCaP, VCaP and 22Rv1 cells, our data showed that theranekron administration remarkably decreased the expression level of AR protein in a dose-dependent manner (Figure 2). These data suggest that theranekron has a potent regulatory role on AR protein levels in AR expressed PCA cells.
In addition, we examined the changes in cell cycle-related cyclin A2, cyclin B1 and cyclin E1 protein levels by immunoblotting, based on the effect of theranekron on cell viability. Cyclin proteins regulate the transition between the phases of the cell cycle by activating cyclin-dependent kinase (CDK) enzymes. Cyclin A2 protein activates the CDK2 kinase and promotes the G1/S and G2/M phase transitions in the cells. Cyclin B1 regulates the transition from G2 phase to mitosis. Cyclin E1 is essential for the G1 phase progression and entry into S phase in mammalian cell division cycle. Our data indicated that theranekron treatment markedly reduced the cyclin A2, B1 and E1 expression in a dose-dependent manner in all tested androgen-dependent PCa cells (Figure 2). These results suggest that theranekron exhibits the anticancer effect by inducing cell cycle arrest on LNCaP, VCaP and 22Rv1 cells.

Finally, we examined the effect of theranekron on 3D tumor formation of PCa cells. Anchorage independent growth is a hallmark of carcinogenesis. The most important limitation of studies in vitro monolayer culture systems are the insufficient 3D cell interactions and inability to mimic in vivo models of tumor formation exactly. In addition, since the distribution of bioactive compounds on cells in monolayer culture systems is 2-dimensional, in vivo test results are mostly failed. 3D culture models created with soft agar colony formation are one of the models that best mimic in vivo tumor forms. For this aim, we performed the 3D PCa formation for LNCaP, VCaP and 22Rv1 cells, and then we tested the effect of theranekron on tumor progression and tumor volumes. Our findings showed that theranekron significantly reduced the tumor formation for all tested PCa cells in a dose-dependent manner (Figure 3a, b). Collectively these results suggest that theranekron has a potent antitumorigenic activities on PCa cells by regulating the androgenic signal mechanisms and leading to cell cycle arrest. These results revealed new biochemical effects of theranekron on PCa cells.

**Study Limitations**

In this study, the anticancer effect of theranekron on PCa cells was investigated in vitro. To investigate the effect of theranekron on PCa cells in more detail, further in vivo studies should be performed.

**CONCLUSION**

The present study suggests that the usage of Theranekron® is not only effective on animal tumors in the veterinary field but can also offer effective therapeutic results on human tumors.

**Acknowledgment**

We thank Suleyman Demirel University - Innovative Technologies Application and Research Center. We thank Fahri Saatcioglu (Department of Biosciences, University of Oslo, Norway) for providing the human prostate adenocarcinoma cell lines LNCaP, VCaP and 22Rv1.

**Ethics approval**

This study does not require any ethical permission.

**Data availability**

The data generated in this study are available upon request from the corresponding author.

**Author Contributions**

Y.E. initiated and directed the project, designed, and conducted the experiments, analyzed, and interpreted the results, and wrote the manuscript. H.K.D. and D.C. assisted in experimental studies. All correspondence and requests for materials should be addressed to Y.E. All author has given final approval of the submitted manuscript.

**Conflict of Interest**

The author(s) declare that they have no conflict of interest.

**Financial Support**

This study was supported by Suleyman Demirel University internal funds (TSG-2021-8302, TAB-2020-8253).

**REFERENCES**


**Tables**

**Table 1.** The values of the effect of theranekron on cell viability on LNCaP, VCaP and 22Rv1 cells. Data are represented as the mean and standard deviation (±) of three independent repeats.

<table>
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<td>LNCaP</td>
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<tr>
<td>VCaP</td>
<td>61.256 ± 3.82</td>
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<td>22Rv1</td>
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<td>50.691 ± 5.31</td>
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**Figures and Figure Legends**
Figure 1. Theranekron treatment decreased the cell viability of androgen-dependent PCa cells. 

a. Microscopic images of theranekron applied LNCaP, VCaP and 22Rv1 cells. Scale bars indicate 5µm. 

b. Cells were treated with vehicle, 12.5 and 25 µg/ml theranekron for 24 hours and viability of cells were measured by WST-1 assay. Data represented as mean ± SE of three independent experiments made in three replicants (*p<0.05, #p<0.01).
Figure 2. Evaluation of the effect of theranekron on AR and cyclin A2, B1 and E1 proteins. PCa cells were seeded in 6-well plates and treated with a vehicle, 12.5 and 25 µg/ml theranekron for 24 hours. Total protein was isolated from the cell pellets as described in the material-method section and then AR, cyclin A2, cyclin B1 and cyclin E1 proteins were examined by immunoblotting studies. Beta-actin was used as a loading control. The densitometry of protein bands was calculated by Image Studio™ Lite (LI-COR®) and normalized against the control group and presented under the immunoblotting results.

<table>
<thead>
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<th>LNCaP</th>
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**Graphical representation of densitometry results (n=3).** The control group was set to 1. (*p<0.05, #p<0.001).
Figure 3. The effect of theranekron on 3D tumor formation of PCa cells. a. Anchorage-independent tumor formation of PCa cells was examined by soft agar colony formation assay as described in the material-method section. b. % inhibition of colonial growth was measured by ImageJ software. Three independent biological and three technical repeats per experiment were performed. Representative images are shown. (*p<0.05, #p<0.005).

Graphical Abstract

Androgen dependent PCa cells

Theranekron®

Cell Cycle Arrest
Inhibits the Androgenic Signal
Decrease the Cell Viability
Block the 3D tumor formation

LNCaP (CRL-1740™)
VCaP (CRL-2876™)
22Rv1 (CRL-2505™)