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ORIGINAL ARTICLE

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

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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 treatment success. Therefore, there is a need for new approaches as powerful resources for use in alternative or supportive therapeutic strategies for anticancer therapeutics. Theranekron® is a commercially available alcoholic extract of *Tarantula cubensis*. Recent studies have shown the potent anticancer effect of theranekron in human tumors, including PCa. Herein, we comparatively examined the antiproliferative activity of theranekron and its biochemical action on androgenic signaling 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-1740TM), 22Rv1 (CRL-2505TM), and VCaP (CRL-2876TM) were used to evaluate the effect of theranekron *in vitro*. The impact of theranekron on cell viability was evaluated using a WST-1-based viability test. Its impact on AR, cyclin A2, cyclin B1, and cyclin E1 was examined by immunoblotting. To test the anti-malignant effect of theranekron on 3D tumor formation of PCa cells, soft agar assay was used.

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. In addition, Theranekron administration strongly limited the 3D tumor formation of LNCaP, 22Rv1, and VCaP cells.

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 acquired resistance to chemotherapeutics.

Key words: Androgen receptor, anti-cancer, cell-cycle, theranekron, prostate cancer, venom

INTRODUCTION

Cancer affected 17 million people in 2018 and led to the death of 9.6 million people worldwide.[1] Prostate cancer (PCa) is a significant health problem and is the second most common cancer type in men worldwide. Surgical intervention, cryotherapy, chemotherapy, and androgen deprivation therapy are frequently preferred for treating PCa.[2,3] However, both resistance to chemotherapeutics and conversion of PCa to castration-resistant PCa substantially limit treatment success.[4]

Animal venoms have a complex rich mixture of various bioactive molecules and thereby 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 anticancer therapeutics, and its biochemical activities are being studied on a large scale by numerous research groups.[5,6]

Tarantula cubensis, also known as the Cuban tarantula, is a large arachnid from the family Theraphosidae.[7] Theranekron® is a commercially available alcoholic extract of *Tarantula clabensis* and is widely used in veterinary medicine 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,

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wound healing, and anticancer well.[9,10] Moreover, resorptive, regenerative, antiphlogistic, and demarcative effects have been reported in proliferative and necrotic tissues.[11,12] The antitumor properties of theranekron have been reported in canine mammary tumors and in *in vitro* human cancer models.[13,14] Very recent *in vitro* studies have focused on the effects of theranekron on different cancer models, including breast, lung, osteosarcoma, and prostate.[15] Our previous study showed that the androgen-sensitive human prostate adenocarcinoma cell line LNCaP was more sensitive to Theranekron than the normal prostate cell line PNT1A.[13] In the present study, we investigated the effects of theranekron on LNCaP, VCaP, and 22Rv1 cells, known as androgen-dependent PCa cell lines.

Herein, we comparatively examined the antiproliferative activity of Theranekron® and investigated its biochemical action on androgenic signaling and cell cycle-related cyclin proteins by immunoblotting. In addition, we tested the action of Theranekron on anchorage-independent cell growth of androgen-dependent PCa cells 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 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 Medium were obtained from Capricorn Scientific. Theranekron® was provided by Richter Pharma AG, Wels, Austria.

Rabbit polyclonal anti-cyclin A2 (#91500) (1:2000), anti-cyclin B1 (#12231) (1:2000), and anti-cyclin E1 (#20808) (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 by 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

The human androgen-sensitive prostate adenocarcinoma cell lines LNCaP (CRL-1740™), 22Rv1 (CRL-2505™), and VCaP (CRL-2876™) were obtained from American Type Tissue Culture. LNCaP and 22Rv1 cells were cultured in RPMI 1,640. VCaP cells were propagated in DMEM. All cell culture media were enriched with 10% FBS, 2 mM L-glutamine, and 5 mg mL^{-1} penicillin/streptomycin (Capricorn-Scientific). Cultured cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 °C.

Cell viability assay

Cells were seeded in 96-well plates (10,000 cells/well) and grown for 24 h. The cells were then treated with theranekron in various doses for 48 h. The WST-1 cell viability assay (Takara) was performed according to the manufacturer's instructions. The absorbance was determined at 450 nm with 600 nm as the reference wavelength using a microplate spectrophotometer (BioTek, Epoch 2). Average absorbance values were calculated, and viability rates are presented in the graph as a percentage fold change. IC_{50} values of Theranekron® were determined using GraphPad Prism 5 software.

Western blotting

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and centrifuged at 14,000 rpm for 20 min at 4 °C. The insoluble phase was removed and the supernatant was collected. The concentration of total protein was determined by the bicinchoninic acid (BCA) assay (Takara). Protein (30 µg) was used in immunoblotting studies. Samples were denatured in 4x Laemmli buffer at 70 °C for 15 min and separated on hand-cast polyacrylamide gels. Separated proteins were transferred to an 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 h at room temperature, and then primary and secondary antibodies were applied for 2 h at room temperature. Target proteins were monitored using enhanced Clarity™ chemiluminescence (ECL) solution (Bio-Rad) in ChemiDoc XRS+ (Bio-Rad). The densitometry of protein bands was calculated using Image Studio™ Lite (LI-COR®).

Soft agar assay

The soft agar colony formation assay was adapted according to Borowicz.[16] Equal volumes of 2 DMEM and 2 RPMI-1640 with 20% FBS and sterile 1.2% low-melting agar were gently mixed and added to a 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 the solidified bottom agar layer. Theranekron® was applied to the cells, and the images of growing colonies were taken using a Sunny SopTop inverted microscope and an OD400UHW camera system. Colonial growth was quantified by taking from independent areas to 5 images and counting.

Statistical analysis

Data are expressed as means ± standard deviation. Statistical significance was confirmed using paired two-tailed Student's *t*-test, and multiple comparisons of significance were analyzed by one-way ANOVA and Tukey's tests (*$p < 0.05$, #$p < 0.005$).

RESULTS

Theranekron® decreases the viability of human androgen-dependent PCa cells

To investigate the effect of theranekron on the viability of androgen-dependent LNCaP, 22Rv1, and VCaP PCa cells, we performed a WST-1-based cell viability test. Theranekron® doses used in this study were selected according to the results of our previous study with LNCaP cells.[13] For this aim, we treated PCa cells with 12.5 µg/mL and 25 µg/mL doses of theranekron for 24 h and then measured cell viability. Our findings revealed

that theranekron administration significantly decreased the cell viability of all tested PCa cells in a dose-dependent manner (Figure 1a, b). In addition, we determined that VCaP cells were more susceptible to theranekron than LNCaP and 22Rv1 cells.

Theranekron® decreases AR levels and induces cell cycle arrest in PCa cells

To evaluate the action of Theranekron® on cell cycle-related proteins of androgen-dependent PCa cells, we treated 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. We found that theranekron application decreased the expression levels of all tested cyclin proteins in a dose-dependent manner (Figure 2a, b). In addition, we tested androgen receptor (AR) protein levels and 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 3D tumor formation in PCa cells

Most animal model trials fail because bioactivity tests performed in monolayer culture systems are insufficient to mimic real tumor formation and tumor environment. Therefore, we conducted 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 strongly reduced the developing tumor volume in all three PCa cell lines (Figure 3a, b).

DISCUSSION

Currently, a few natural compounds or their synthetic analogs are clinically used against cancer.[17] In particular, spider venoms show potent effects on cancer cells because of their strong bioactive contents. Therefore, they are seen as potential drug candidates because of their anticancer and antinociceptive activities.[18] Numerous spider venoms 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, an active compound of *Lycosa singoriensis* venom, activates mitochondrial cell death signaling in human lung adenocarcinoma, human prostate carcinoma, and colon adenocarcinoma cells.[19]

The commercially available alcoholic extract of *Tarantula cubensis* venom, Theranekron®, is often used in veterinary medicine to treat animal tumors. Therapeutically, Theranekron® has exhibited anticancer, anti-inflammatory, antiphlogistic, demarcative, and wound healing properties in clinical studies.[7]

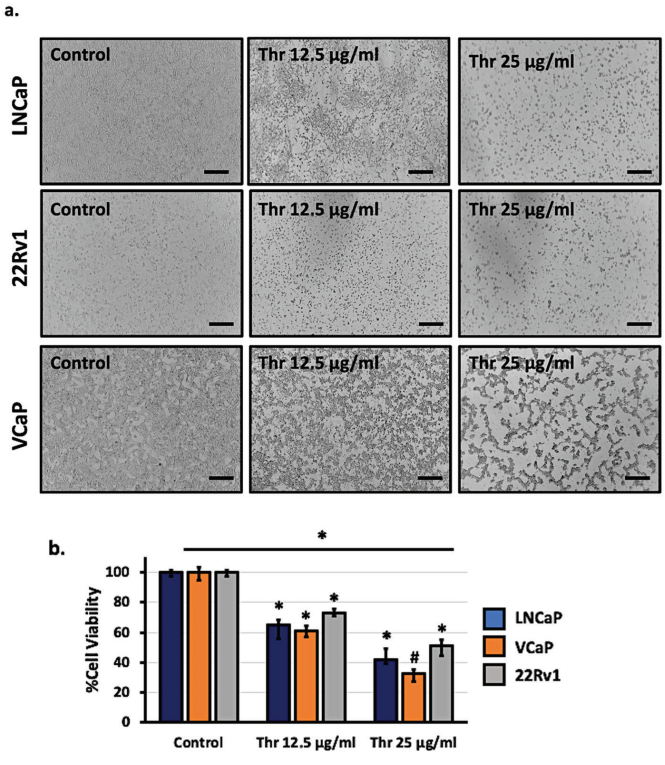

Figure 1. Theranekron® treatment decreased the viability of androgen-dependent PCa cells. a) Microscopic images of Theranekron-applied LNCaP, VCaP, and 22Rv1 cells. Scale bars indicate 5 µm. b) Cells were treated with vehicle, 12.5, and 25 µg/mL Theranekron® for 24 h, and cell viability was measured by WST-1 assay. Data are represented as mean ± SE of three independent experiments performed in three replicants (*p < 0.05, #p < 0.01).

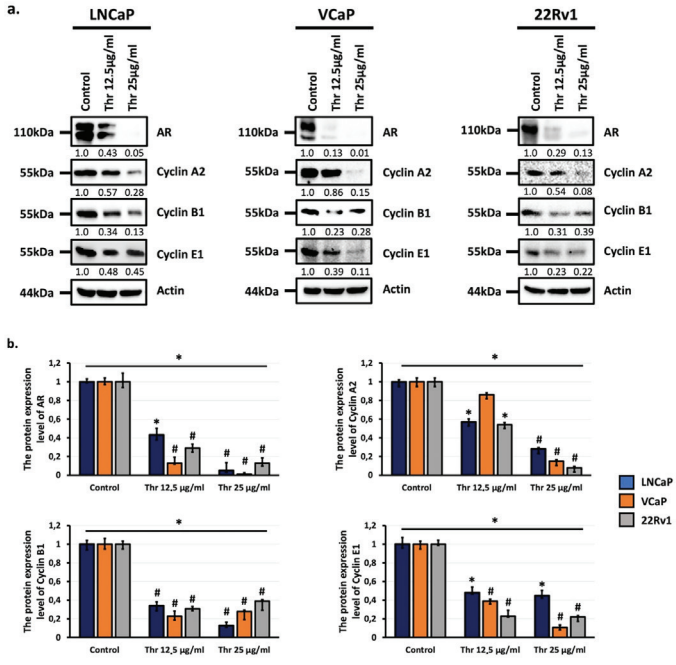

Figure 2. Evaluation of the effect of Theranekron® on AR and cyclin A2, B1, and E1 proteins. a) PCa cells were seeded in 6-well plates and treated with a vehicle, 12.5, and 25 µg/mL Theranekron for 24 h. Total protein was isolated from the cell pellets as described in the Materials and Methods section, and AR, cyclin A2, cyclin B1, and cyclin E1 proteins were examined by immunoblotting. Beta-actin was used as the loading control. The densitometry of protein bands was calculated using Image StudioTM Lite (LI-COR®), normalized against the control group, and presented under the immunoblotting results. b) Graphical representation of the densitometry results (n: 3). The control group was set to 1. (*p < 0.05, #p < 0.001)

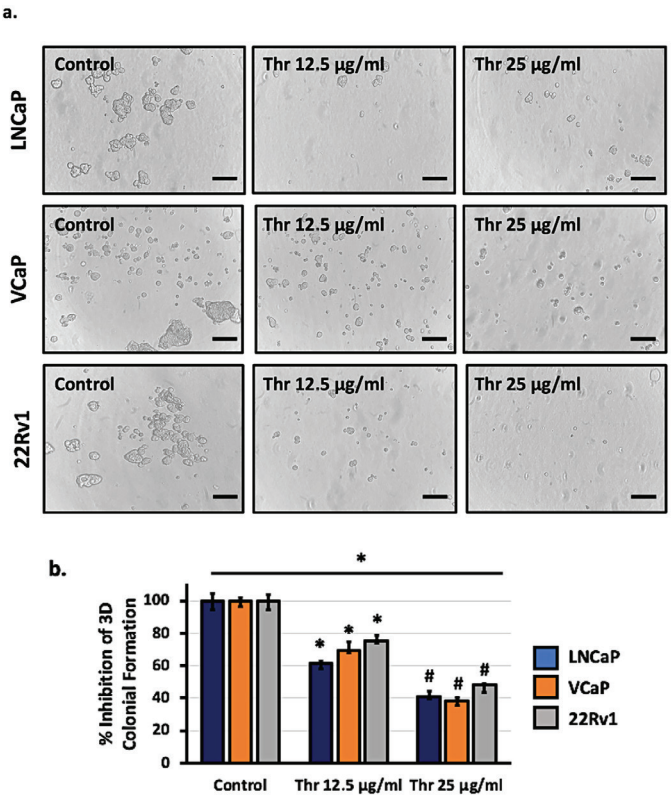

Figure 3. The effect of Theranekron® on 3D tumor formation of PCa cells. a) Anchorage-independent tumor formation of PCa cells was examined using a soft agar colony formation assay, as described in the Materials and Methods section. b) % inhibition of colonial growth was measured using ImageJ software. Three independent biological and three technical repeats *per* experiment were performed. Representative images are shown (*$p < 0.05$, #$p < 0.005$)

Table 1. Values of the effect of Theranekron on cell viability in LNCaP, VCaP, and 22Rv1 cells. Data are presented as the mean and standard deviation (±) of three independent repeats

	Cell viability	
Cell lines	**Thr 12.5 µg/mL**	**Thr 25 µg/mL**
LNCaP	65.256 ± 4.46	42.939 ± 1.96
VCaP	61.256 ± 3.82	35.931 ± 2.32
22Rv1	73.256 ± 3.81	50.691 ± 5.31

In addition, the usage of Theranekron has been reported in endometritis, cutaneous papillomatosis, pododermatitis, and foot and mouth lesions in veterinary medicine.[20-24] Recent studies have focused on the anticancer effect of Theranekron® in human cancer cells.[13,15] Erzurumlu et al.[13] reported that the androgen-dependent PCa cell line LNCaP was more susceptible to the androgen-independent metastatic PCa cell line Du145 and the healthy prostatic cell line PNT1A. Mechanistically, it affects autophagic activity and induces endoplasmic reticulum stress in androgen-dependent PCa cells. In addition, it markedly reduced the epithelial-mesenchymal transition of LNCaP cells.[13]

Herein, we focused on the impact of theranekron in androgen-dependent PCa cells and comparatively investigated the roles of androgenic signaling, cell cycle, and therapeutic impact on the 3D tumor formation of PCa cells. First, 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 with LNCaP and 22Rv1 cells (Figure 1). In addition, 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 PCa cell progression. AR protein is induced by androgens in androgen-dependent PCa cells, and the expression of AR target genes is then stimulated through a specialized transcriptional program.[25,26] AR target genes include proto-oncogenic gene products that support prostate tumorigenesis. Therefore, suppression of AR signaling is among the major therapeutic choices developed for PCa. We evaluated the effect of Theranekron® on AR protein levels in LNCaP, VCaP, and 22Rv1 cells and found that Theranekron administration remarkably decreased the expression level of AR protein in a dose-dependent manner (Figure 2). These data suggest that Theranekron plays a potent regulatory role in AR protein levels in AR-expressing 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 phases of the cell cycle by activating cyclin-dependent kinase (CDK) enzymes.[27] Cyclin A2 protein activates CDK2 kinase and promotes G1/S and G2/M phase transitions in cells.[28] Cyclin B1 regulates the transition from the G2 phase to mitosis.[29] Cyclin E1 is essential for G1 phase progression and entry into the S phase in the mammalian cell division cycle.[30,31] Our data indicated that Theranekron treatment markedly reduced 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 an anticancer effect by inducing cell cycle arrest in 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.[32] The most important limitations of studies on *in vitro* monolayer culture systems are the insufficient 3D cell interactions and the inability to mimic *in vivo* models of tumor formation exactly.[33,34] In addition, because the distribution of bioactive compounds on cells in monolayer culture systems is two-dimensional, *in vivo* test results have 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 purpose, we performed 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 tumor formation in all tested PCa cells in a dose-dependent manner (Figure 3a, b). Collectively, these results suggest that Theranekron® has potent antitumorigenic activities on PCa cells by regulating androgenic signaling 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 use of Theranekron® is not only effective on animal tumors in the veterinary field but can also offer effective therapeutic results on human tumors.

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Ethics

Ethics Committee Approval: This study does not require any ethical permission.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Y.E., Design: Y.E., Data Collection or Processing: Y.E., Analysis or Interpretation: Y.E., Literature Search: Y.E., H.K.D., D.Ç., Writing: Y.E.

Conflict of Interest: No conflict of interest was declared by the authors.

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