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A. INTRODUCTION

Early stage prostate cancer depends heavily on androgen signaling for growth and clonal expansion. In the previous report, we have demonstrated the combination of selenium, which down-regulates androgen receptor, and finasteride, a 5α -reductase inhibitor, has a synergistic effect in inhibiting the growth of prostate cancer cells, suggesting this novel combination is a promising strategy for preventing prostate cancer. We have also demonstrated that FOXO1A plays a critical role in mediating apoptosis induction by selenium. In the second grant period, we have focused our effort on the following areas: 1) studying the impact of the selenium and finasteride combination on androgen signaling; 2) Identifying the pro-apoptotic target genes of FOXO1 that are induced by selenium; 3) studying the potential AR antagonistic effect of finasteride. The last was not proposed in the original application. But we strongly believe this is a topic worthwhile pursuing since a thorough understanding of the activities of finasteride is important for its use in clinical practices.

B. BODY

Task 1. Evaluate the efficacy of selenium and finasteride combination on cell growth in cell culture

Synergy between selenium and finasteride in suppressing AR transactivation

To thoroughly examine the impact of the combination on the transcriptional activity of AR, we

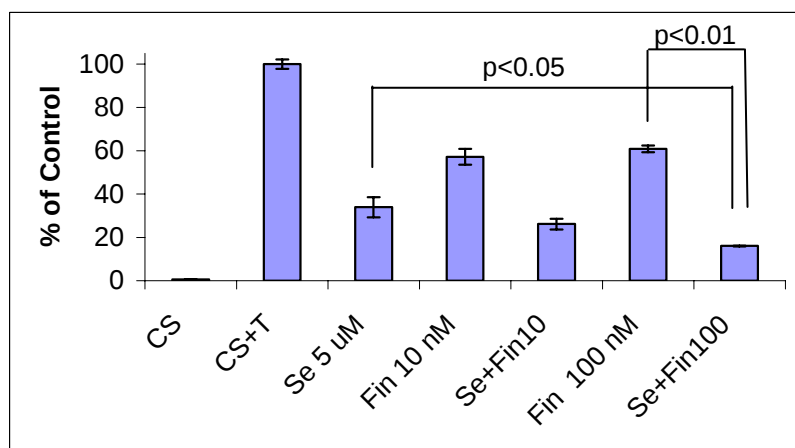


Fig. 1. The effect of finasteride and selenium on AR transactivation. CS, charcoal-stripped serum. T, testosterone (10 nM).

transiently transfected LNCaP cells with an ARE-luciferase reporter construct. This construct contains three repeats of the ARE region ligated in tandem to the luciferase reporter gene. Cells were transfected in bulk to eliminate the variations in transfection efficiency. The transfected cells were then split into equal aliquots and plated in triplicate onto 6-well plates in phenol-red

free RPMI 1640 medium containing 10% charcoal-stripped FBS and 0 or 10 nM

testosterone. Cells were treated with finasteride alone (at 10 or 100 nM) for 48 hr, or methylseleninic acid (MSA) alone (at 5 μ M) for 6 hr, or their combination. At the end of treatment, cells were lysed with 1X Passive Lysis Buffer (Promega) and analyzed for luciferase activity with the use of the Luciferase Assay System (Promega). Total protein concentration were determined in the cell lysate and used for normalizing the luciferase activity. As shown in Fig. 1, MSA treatment decreased AR transcriptional activity by 64%, whereas finasteride at 10 nM reduced by 40%. Their combination led to a more pronounced suppression of 74%. Elevating the finasteride concentration to 100 nM did not produce further reduction of AR activity. However, when combined with MSA, a very dramatic reduction of greater than 80% was observed. The difference was statistically significant ($p<0.05$).

In addition to the reporter gene assay, we also examined the impact of selenium and

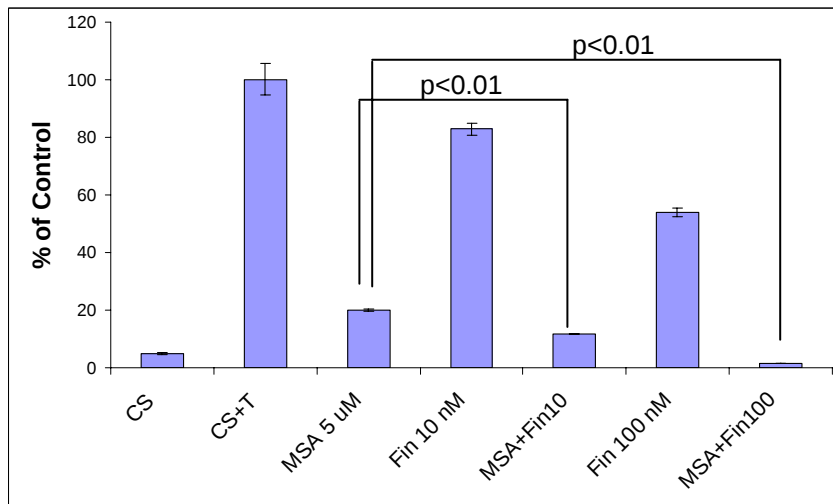


Fig. 2. Suppression of PSA mRNA by MSA and finasteride.

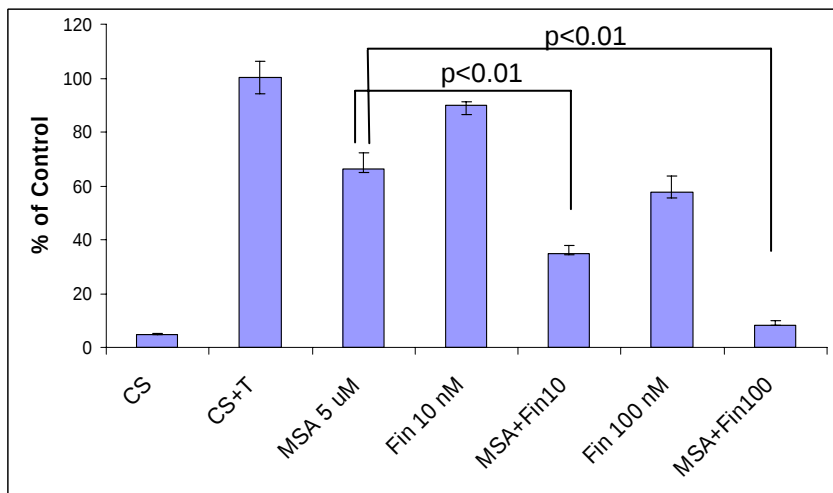


Fig. 3. Suppression of KLK2 mRNA by MSA and finasteride.

were generally smaller (Fig. 4)

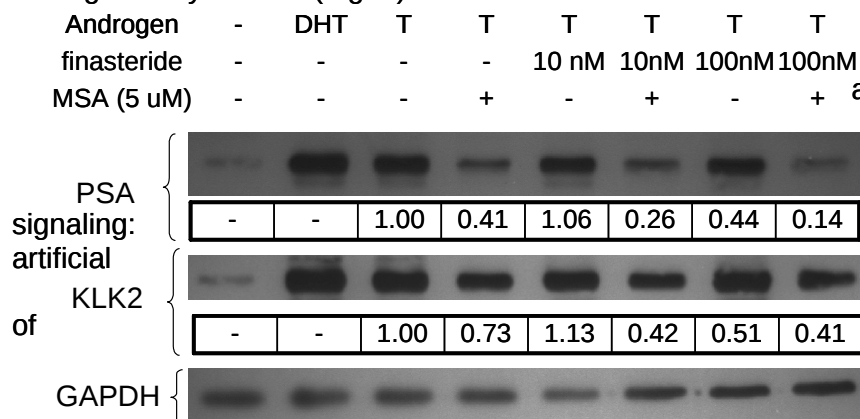


Fig. 4. Suppression of PSA and KLK2 protein expression by MSA and finasteride. Quantitative analysis was performed by volume densitometry. The results were normalized by the respective GAPDH intensities, and expressed as fold relative to the control.

the impact of selenium and finasteride on expression of prostate specific antigen (PSA), and Kallikrein 2 (KLK2), two well known targets of AR. LNCaP cells were treated with finasteride, or MSA, or the combination, as described above. RNA and total protein were extracted and used for real-time RT-PCR and western blotting, respectively. As shown in Fig. 2, finasteride reduced PSA transcript in a concentration dependent manner. MSA lowered

PSA expression very efficiently. However, when it is used in combination with finasteride, the suppression was further enhanced. PSA mRNA was barely detectable when 100 nM finasteride was combined with MSA. The expression of KLK2 mRNA was modulated in a nearly identical manner. The changes in mRNA expression were confirmed at the protein level by Western analysis, although the magnitudes of change

In summary, we have employed two different approaches to examine the impact of selenium and finasteride on the genotropic actions of AR one by the use of a reporter construct with contains 3 AREs upstream the luciferase gene, and the other by studying the endogenous AR target genes. The data obtained by these two approaches

are in excellent agreement with each other; both suggest a synergistic interaction between selenium and finasteride in suppressing AR signaling.

Expanded Task 4. Determine whether selenium affects the transactivation activity of FOXO1A

Identification of FOXO1A targets modulated by selenium

In the last report, we demonstrated that selenium induces the transactivation activity of FOXO1A (Task 4), and that induction of FOXO1A is critical for apoptosis induction by selenium (Task 5). Inspired by these observations, we extended this task to identify which of the known

	Control	1 hr	2 hr	3 hr	6 hr	16 hr	24 hr
Bim							
GAPDH							
Fold	1.0	0.75	0.84	0.58	1.45	1.99	2.51
TRAIL							
GAPDH							
Fold	1.0	1.18	4.00	2.10	2.25	1.94	1.80
TRADD							
GAPDH							
Fold	1.0	1.22	1.47	1.06	1.23	1.64	1.88

Fig. 5. Induction of FOXO1A targets by selenium.

that apoptosis induction of selenium is mediated by a subset, but not all, of the pro-apoptotic targets of FOXO.

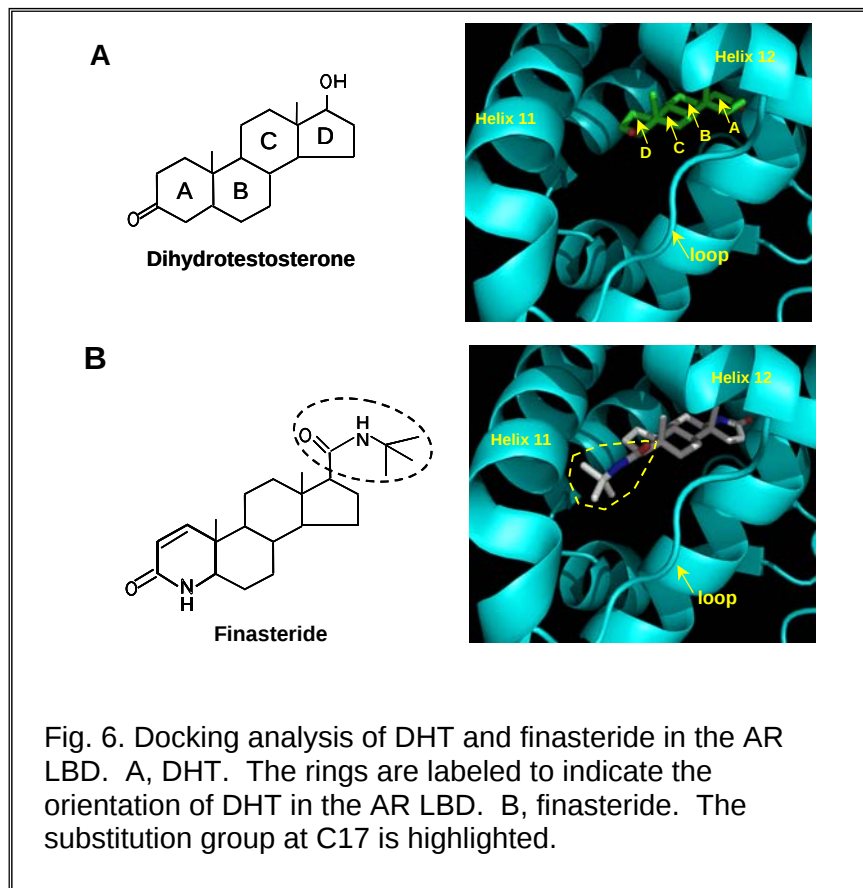
There are two major cell death signaling pathways, one triggered through death receptors (the extrinsic pathway), and the other through the mitochondria (the intrinsic pathway). Identification of the FOXO targets that are induced by selenium provides us with insights into the death signaling pathways modulated by selenium. A signature of the intrinsic pathway is the release of cytochrome C from the mitochondria, which is regulated by the Bcl-2 family of proteins. A pro-apoptotic member of the Bcl-2 family, Bim functions by antagonizing the actions of the anti-apoptotic Bcl-2 and Bcl-X_L. Both TRAIL and TRADD are associated with the extrinsic pathway. By inducing Bim, TRAIL, and TRADD, selenium could activate both the intrinsic and the extrinsic pathway. This is consistent with previous findings(8-11).

New Task. To study the potential AR antagonistic activity of finasteride

Although initially designed as an inhibitor of 5 α -reductase, finasteride has been found to have other biochemical effects in addition to blocking 5 α -reductase. In a study by Long et al (12), it was suggested that finasteride might compete with DHT for binding to AR. A second independent study showed similar anti-androgenic effect for both finasteride and dutasteride (13). Although enticing, both studies cultured cells in charcoal-stripped serum, which is known

to contain a residual, but still significant amount of testosterone. Therefore the role of 5 α -reductase inhibition cannot be totally ruled out. We decide to study the potential AR antagonistic activity using an improved experimental design. The information obtained could have important clinical implications for the use of finasteride as a chemopreventive agent. It has been reported that 35% of alleles in the US population carry mutations in the type II 5 α -reductase gene, encoding variants of the enzyme with a low affinity for finasteride (14). If finasteride is shown to be an AR antagonist, it would suggest that it might be used as an anti-androgen in patients carrying a 5 α -reductase gene with a low affinity for it.

Molecular modeling analysis



To test the possibility that finasteride could bind directly to AR, we performed a computational exercise called docking analysis. This was done through the collaboration with Drs. Yu Xue and Matthew Redinbo at the Department of Chemistry, University of North Carolina at Chapel Hill. Docking analysis is a commonly used computational tool in drug design and discovery, and is well suited for predicting ligand conformation and orientation within the binding site of a protein receptor. The 3D structure of the AR ligand binding domain (LBD) has been determined previously by x-ray crystallography. As shown in Figure 6, finasteride fits

just as well as DHT in the ligand-binding pocket of AR. The docking analysis also suggests that finasteride is a potential AR antagonist. The activation function (AF2) region of AR comprises of a shallow hydrophobic groove on the surface of the LBD, formed by residues from helices H3, H4, H5, and H12. This region, which is formed only in the presence of agonistic ligands, such as DHT, acts as a recruitment surface for coactivators via specific protein-protein interaction. This ligand-dependent nature of AF2 is determined by the positioning of H12, which in turn is influenced profoundly by the side chain of the ligand. Since finasteride possesses a bulky and more hydrophobic substitution group at position C17, it may affect the position of the loop region between H11 and H12. This consequently could prevent H12 from adopting the proper position for interacting with the coactivators and therefore confer AR antagonism.

Competitive ligand binding assay

To determine experimentally whether finasteride could bind to AR, we employed a whole cell, competitive ligand-binding assay. LNCaP cells were plated in triplicate in 24-well plates in complete medium for 48 hr to reach 80% confluency. Cells were then switched to phenol-red free medium plus 0.2% AlbuMax and hormone-starved for 24 hr. [3 H]-R1881, a synthetic DHT analog, was added to the medium in increasing concentrations (from 0.06 nM to 1 nM), in the presence of vehicle alone, 200-fold molar excess of unlabeled R1881, or 10 nM finasteride. Following a 3-hr incubation at 37°C, the binding reaction was stopped by washing the cells 3 times with ice-cold PBS. Cells were lysed in 1X Passive Lysis Buffer (Promega) for scintillation counting. The specific binding was calculated by subtracting the non-specific binding (i.e. the reading obtained in the presence of 200-fold excess of cold R1881) from the total binding. The result was analyzed by Scatchard plot and is shown in Figure 7. Finasteride had little effect on the ligand binding capacity of AR, which is reflected by Bmax (385.3 cpm vs 423.2 cpm). However, the dissociation constant (K_D), which indicates the concentration at which 50% of the receptors are occupied by the radioligand, was increased from 87.8 pM to 307.5 pM by finasteride. The result suggests that finasteride could compete with androgen for binding to AR.

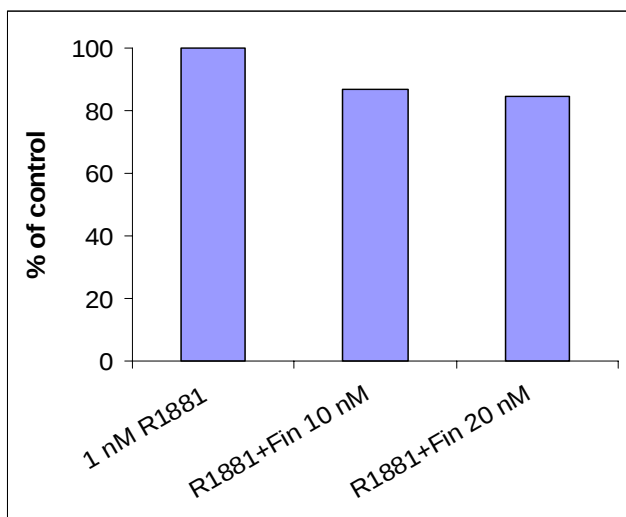
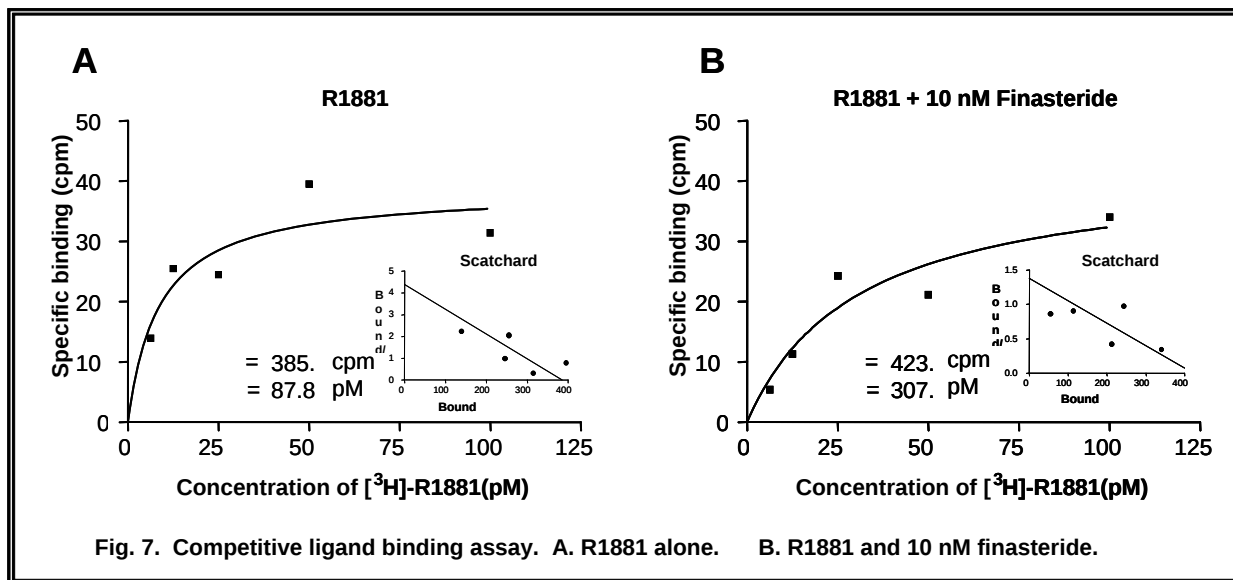


Fig. 8. Potential AR antagonistic activity of finasteride.

AR antagonistic effect of finasteride

We have demonstrated that finasteride could compete with androgen for binding to AR. However, it remains unclear how such binding would affect the activity of AR. To circumvent this problem, we cultured LNCaP cells in a steroid hormone-defined medium containing phenol-red free RPMI 1640, 0.2% AlbuMax (Invitrogen), and supplemented with 1 nM DHT. This medium has no testosterone, but since it

contains a known amount of DHT, the contribution of 5 α -reductase block is completely taken out of the equation. Cells were transiently transfected with the ARE-luciferase reporter construct, as described above. Following transfection, the cells were plated in triplicate onto 6-well plates and treated with 0, 10, or 20 nM finasteride for 16 hours. Cells were then lysed and luciferase activity assay was performed. As shown in Fig. 8, in the condition that was designed specifically to study potential antagonistic effect, finasteride reduced the transactivation activity of AR. The effects were small, but consistent. We are current conducting more experiment to use a wider range of finasteride concentration.

C. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that the combination of MSA and finasteride has a synergistic effect in suppressing androgen signaling
- Identified several pro-apoptotic targets of FOXO that were induced by selenium. This provides insight into the death signaling pathways activated by selenium. These genes could serve as biomarkers to monitor selenium response in future clinical practices.
- Through molecular modeling, competitive ligand binding, and reporter gene analyses, demonstrated that finasteride has antagonistic activity against androgen receptor.

D. REPORTABLE OUTCOMES

Publication

Haitao Zhang, Dian Yao, Yan Dong, and Clement Ip. Apoptosis induction by selenium is mediated by FOXO1A. Manuscript in preparation.

Haitao Zhang, Dian Yao, Felicia Parker, and Clement Ip. Synergistic interaction between selenium and finasteride in prostate cancer chemoprevention. Manuscript in preparation.

Presentation

1. AACR Centennial Meeting, Los Angeles, CA, April 15-18, poster presentation, "Combining selenium and finasteride for prostate cancer chemoprevention".
2. The first international symposium on prostate cancer, Jilin University, Changchun, Jilin, China, July 22-25, 2007, oral presentation, "Translational research of selenium and finasteride in prostate cancer prevention".
3. DOD Innovative Minds in Prostate Cancer Today meeting, Atlanta, GA, Sept 5-8, poster presentation, "Combining selenium and finasteride for prostate cancer chemoprevention".
4. Tulane University, New Orleans, Louisiana, Sept 11, 2007, invited speech, "Translational research of selenium and finasteride in prostate cancer prevention".

Funding applied

1. American Cancer Society Research Scholar Grant, "Enhancing the Chemopreventive Efficacy of Finasteride by Selenium (H. Zhang, PI)", re-submitted April 2007.
2. National Institute of Health P01, "Translational research of finasteride and selenium prevention of prostate cancer (H. Zhang, Co-Project Leader)", funded in September, 2007.

E. CONCLUSIONS

We have successfully demonstrated that the combination of selenium and finasteride synergistically suppresses androgen signaling. The changes in AR-regulated genes, PSA and KLK2, could be detected in both mRNA and protein levels. This confirms the use of these AR targets to monitor the responsiveness to the combination in future clinical practices.

In the last report, we have demonstrated that FOXO1A plays a critical role in mediating apoptosis induction by selenium. We have now identified several pro-apoptotic targets of FOXO1 induced by selenium. Identification of these genes could further our understanding of the death signaling pathways activated by selenium. In addition, these genes could serve as biomarkers to monitor responsiveness to selenium.

The current study provides evidence that finasteride, in addition to its ability to inhibit 5 α -reductase, has AR antagonistic activity. It has been shown 35% of alleles in the US population carry mutations in the type II 5 α -reductase gene, encoding variants of the enzyme with a low affinity for finasteride. The information would suggest individual with these mutations could still benefit from finasteride treatment.

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