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Award Number:

W81XWH-05-1-0598

TITLE:

New Strategy for Prostate Cancer Prevention Based on Selenium Suppression of Androgen Receptor Signaling

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REPORT DATE:

April 2010

TYPE OF REPORT:

Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☒ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
25-04-2010	**Final**	**15 Sep 2005-31 Mar 2010**

4. TITLE AND SUBTITLE

New Strategy for Prostate Cancer Prevention Based on Selenium Suppression of Androgen Receptor Signaling

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0598

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Zhang, Haitao

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Tulane University Health Sciences Center

New Orleans, LA 70112

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research And Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this project, we investigated the potential of targeting the androgen receptor and the 5^{α}-reductase simultaneously in prostate cancer prevention. We report that this combined androgen receptor blockade approach produced a synergistic effect on tumor growth inhibition by in vitro and in vivo. We have also identified that methylseleninic acid (MSA) activates FOXO1 and FOXO1 activation plays an important role in mediating apoptosis induction and androgen receptor suppression by MSA. In summary, the research support by this grant suggests a novel and effective approach for managing early stage prostate cancer and contributes to a better understanding of the anticancer effects of MSA.

15. SUBJECT TERMS
selenium; 5α-reductase inhibitor; prostate cancer; chemoprevention; androgen receptor; FOXO1

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	40	19b. TELEPHONE NUMBER *(include area code)*

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A. INTRODUCTION

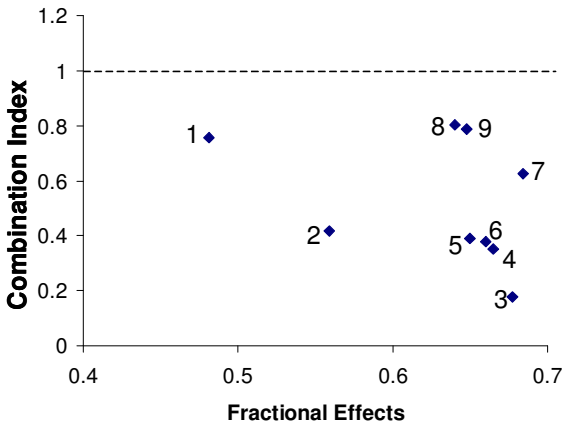
Androgen plays an important role in prostate carcinogenesis. Testosterone is the major androgen in circulation; it is converted to the more potent dihydrotestosterone in the prostate by the enzyme 5α-reductase. The Prostate Cancer Prevention Trial (PCPT) demonstrated that treatment with finasteride, an inhibitor of 5α-reductase, reduced prostate cancer incidence by 25%. Selenium, on the other hand, is shown to reduce prostate cancer risk by 50% by the Nutrition Prevention of Cancer (NPC) trial. In vitro studies have shown that selenium suppresses androgen signaling by downregulating expression of the androgen receptor (AR). This project is consists of two specific aims: 1). To evaluate the combined use of selenium and a 5α-reductase inhibitor in preventing prostate cancer; 2). To investigate the role of FOXO1 in mediating the anticancer effect of selenium. This report summarizes our research findings for the entire period of the study. Several new tasks have been added during the course of this study.

B. BODY

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

Synergy of finasteride and MSA in growth inhibition of prostate cancer cells

To determine the combinatory effect of finasteride and MSA, LNCaP cells were grown in phenol-red free RPMI 1640 containing 10% charcoal-stripped serum and 1 nM testosterone to replete the hormone-deprived medium. After allowing cells to attach for 48 hr, we began treatment with 0, 2.5, 5 or 10 nM of finasteride and 0. 2.5, 5 or 10 μM of MSA in a 4 x 4 factorial design. The total of 16 cultures were distributed as follows: 1 untreated control culture, 3 escalating MSA dose cultures, 3 escalating finasteride dose cultures, and 9 finasteride/MSA combination cultures. The MTT cell growth data were analyzed by the Calcusyn software (Biosoft). This program uses the median-effect



1: Finasteride (2.5 nM) + MSA (2.5 μM)
2: Finasteride (5 nM) + MSA (2.5 μM)
3: Finasteride (10 nM) + MSA (2.5 μM)
4: Finasteride (5 nM) + MSA (5 μM)
5: Finasteride (5 nM) + MSA (5 μM)
6: Finasteride (10 nM) + MSA (5 μM)
7: Finasteride (2.5 nM) + MSA (10 μM)
8: Finasteride (5 nM) + MSA (10 μM)
9: Finasteride (10 nM)+ MSA (10 μM)

Figure 1. Combination index plot of the synergy between finasteride and MSA on growth inhibition.

principle (1) to delineate the interaction between two drugs. For each dose combination, the program generates a combination index (CI) based on the equation below (2). $(D)_1$ and $(D)_2$ represent the doses of drug 1 and drug 2 in combination which inhibit cell growth by X% based on empirical observations. $(D_x)_1$ and $(D_x)_2$ are the theoretical doses of drug 1 and drug 2, that will achieve X% inhibition if they are used alone. $(D_x)_1$ and $(D_x)_2$ are calculated from the median-effect formula (1) based on the dose curve of each drug.

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1\,(D)_2}{(D_x)_1(D_x)_2}$$

A combination index (CI) of < 1, 1 or > 1 denotes synergism, additivity or antagonism, respectively. Each spot in Fig. 1 corresponds to the combination number shown beneath the plot. All nine combinations produced a CI value of less than 1, suggesting a synergy between finasteride and MSA in cell growth inhibition. The smaller the CI value, the better is the synergistic effect. Combination 3 (10 nM finasteride and 2.5 μM MSA) showed the strongest synergy and produced a 70% inhibition of growth. It is important to appreciate that the combination which offers the strongest synergy may not be the same combination which produces the greatest growth inhibition in terms of absolute value. High doses of finasteride and MSA will completely block cell growth, but this is not the outcome we are looking for. We are trying to find the best performance combination without pushing each drug into the high dose range.

Synergy of finasteride and MSA in apoptosis induction

The previous section demonstrated a synergistic effect of finasteride and MSA in arresting the growth of LNCaP cells by the MTT assay, which measures cell number. A reduction in cell number could be attributed to decreased cell proliferation and/or increased cell death. To study apoptosis induction, LNCaP cells were cultured in a hormone-depleted medium and supplemented with testosterone as described previously, and treated with 10 or 100 nM finasteride for 48 hr, 2.5 μM MSA for 24 hr, or the combination. Apoptosis was quantitated by using the Cell Death Detection ELISA kit (Roche). The method is highly specific and sensitive for quantitation of apoptotic cell death. ΔOD_{405}, defined as the OD_{405} reading of the treated cells minus that of the control, is used to indicate the extent of apoptosis. As shown in Figure 2, MSA alone induced apoptosis by 0.02 OD_{405} units, while finasteride

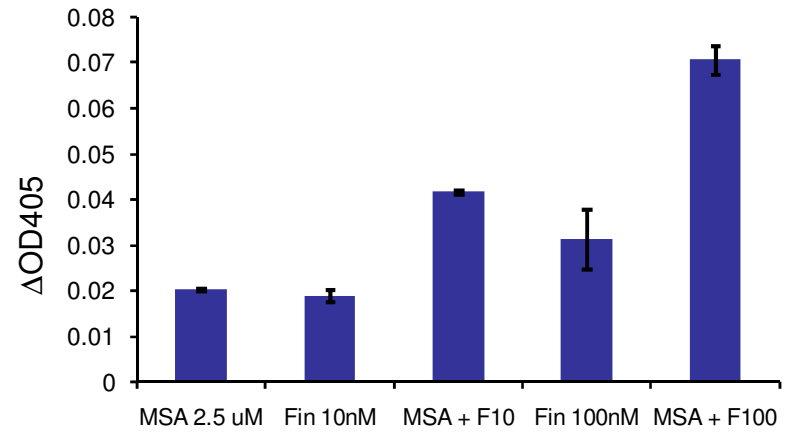


Figure 2. Apoptosis induction by finasteride and MSA. LNCaP cells were cultured in medium containing charcoal-stripped serum and supplemented with 10 nM T, and treated with MSA and/or finasteride for 24 hr. *, P<0.01 than either single treatment, n=3.

at the lower concentration resulted in a similar increase. However, the combination led to an induction of 0.04 units. At the 100 nM dose, finasteride was more effective in apoptosis induction. Once again, the combination with selenium led to a more pronounced induction (Lane 5).

To study the effect of finasteride and MSA on PARP cleavage, a characteristic indicative of caspase activation, LNCaP cells were treated with 0, 50, or 100 nM finasteride for 32 hr, and 0 or 5 μM MSA for an additional 16 hr. Whole cell lysate was prepared and Western blotting was performed using an antibody specific for cleaved PARP (89 kDa), and the result is shown in Figure 3. After 48 hr of treatment, finasteride at 50 and 100 nM increased PARP cleavage by 1.7 and 1.9 fold, respectively (lanes 2 and 3). A similar induction was observed when cells were treated with 5 μM MSA for 16 hr (lane 4). The induction was far more greater when finasteride and MSA were used in combination, led to a more pronounced induction. Together with Figure 2, these results suggest a synergistic interaction between finasteride and MSA in apoptosis induction.

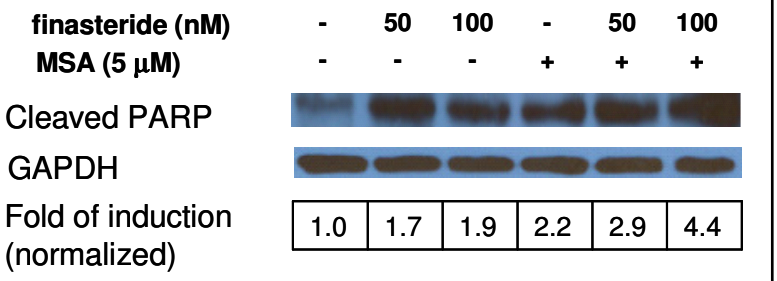


Figure 3. Induction of PARP cleavage by finasteride and MSA.

Augmented suppression of androgen signaling by finasteride and MSA

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

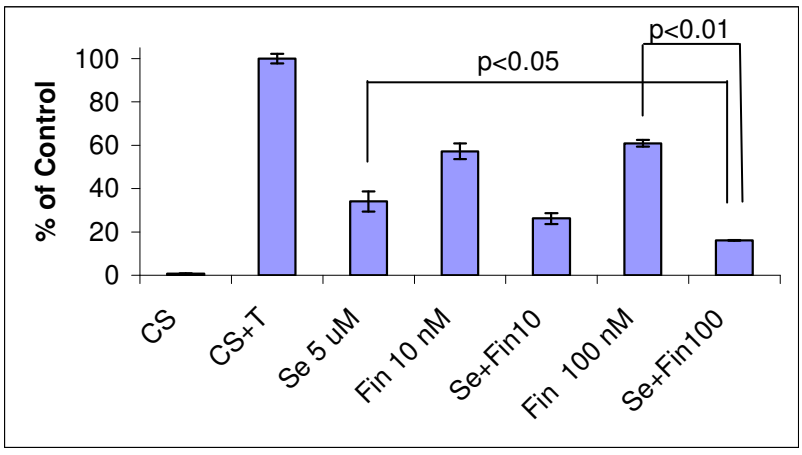


Fig. 4. The effect of finasteride and selenium on AR transactivation. CS, charcoal-stripped serum. T, testosterone (10 nM).

nM) for 48 hr, or methylseleninic acid (MSA) alone (at 5μM) 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. 4, 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 ($p<0.05$).

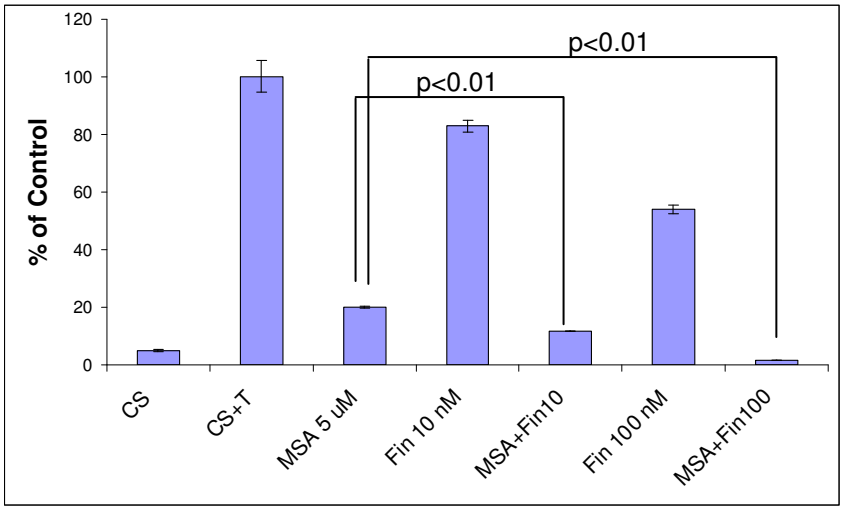


Figure. 5. Suppresion of PSA mRNA by MSA and finasteride.

In addition to the reporter gene assay, we also examined 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. 5, 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 (Fig. 6). The changes in mRNA expression were confirmed at the protein level by Western analysis, although the magnitudes of change were generally smaller (Fig. 7).

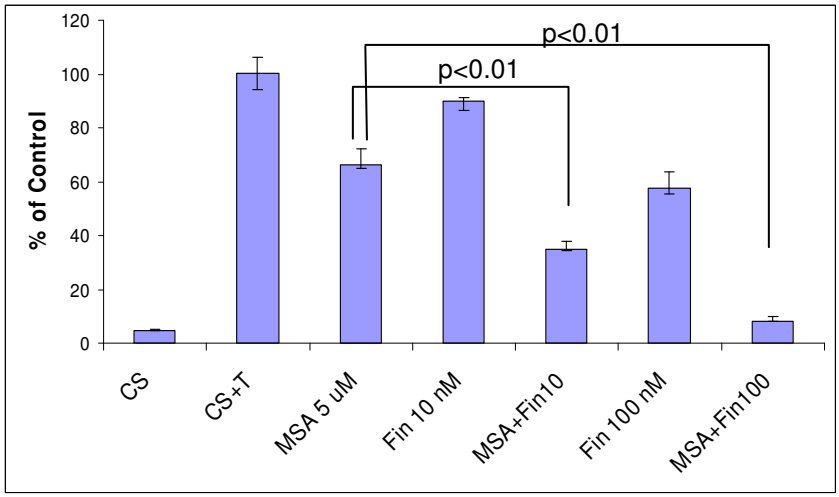


Figure 6. Suppresion of KLK2 mRNA by MSA and finasteride.

In summary, we have employed two different approaches to examine the impact of selenium and finasteride on the genotropic actions of AR signaling: one by the use of an artificial reporter construct with contains 3 AREs upstream of 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.

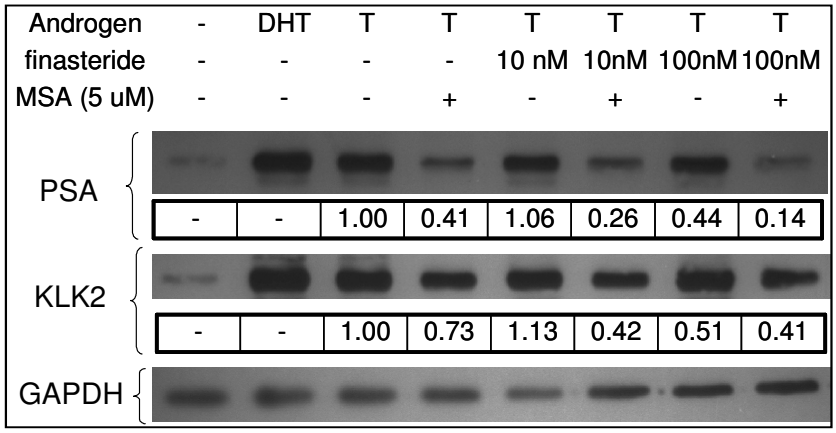


Figure 7. Suppression of PSA and KLK2 protein expression by MSA and finasteride. Quantitative analysis was performed by volume densitometry. The results were normalized by the respective GAPDH intensities, and expressed as fold relative to the control.

Task 2. Determine the optimal dose of finasteride to achieve growth inhibition of tumor xenografts in nude mice. In the original proposal, we proposed to identify an optimal dose of dutasteride for the combination experiments. We have since switched to finasteride and we found in the literature doses of finasteride effective in inhibiting the growth of LNCaP xenografts in nude mice inhibiting LNCaP xenograft (3,4). We chose to use these doses in the following experiments.

Tasks 3. Assess the combinatorial effect of finasteride and selenium on growth of tumor xenografts in nude mice. Based on the literature information, we decided to use finasteride at 5 and 50 mg/kg/day, in combination with MSC at 100 µg/day. For xenografting, $4X10^6$ LNCaP cells were suspended in 50 µl Matrigel (Becton Dickinson Labware) and injected subcutaneously to both sides of the dorsal flank. The Matrigel milieu is required for the formation of tumors in immunodeficient mice (5). Forty-eight mice were randomized to 6 groups, with 8 mice per group (Table 1). MSC and finasteride were administered the day after tumor implantation. Finasteride was prepared in a mixture of 10% ethanol/90% olive oil and given to the mice by oral gavage using a ball-tipped

Table 1. Treatment groups and tumor take rate.

Group ID	# of Mice	MSC (µg/kg)	Fin (mg/kg)	# of tumor	Tumor take rate
Control	8	-	-	16	100%
MSC	8	100	-	16	100%
FL	8	-	5	15	93.7%
FH	7*	-	50	13	92.8%
MFL	7*	100	5	14	100%
MFH	7*	100	50	13	92.8%

*One mouse in each of these groups died accidentally. FL, finasteride, low dose. FH, finasteride, high dose. MFL, MSC plus low dose finasteride. MFH, MSC plus high dose finasteride.

feeding needle. MSC was dissolved in

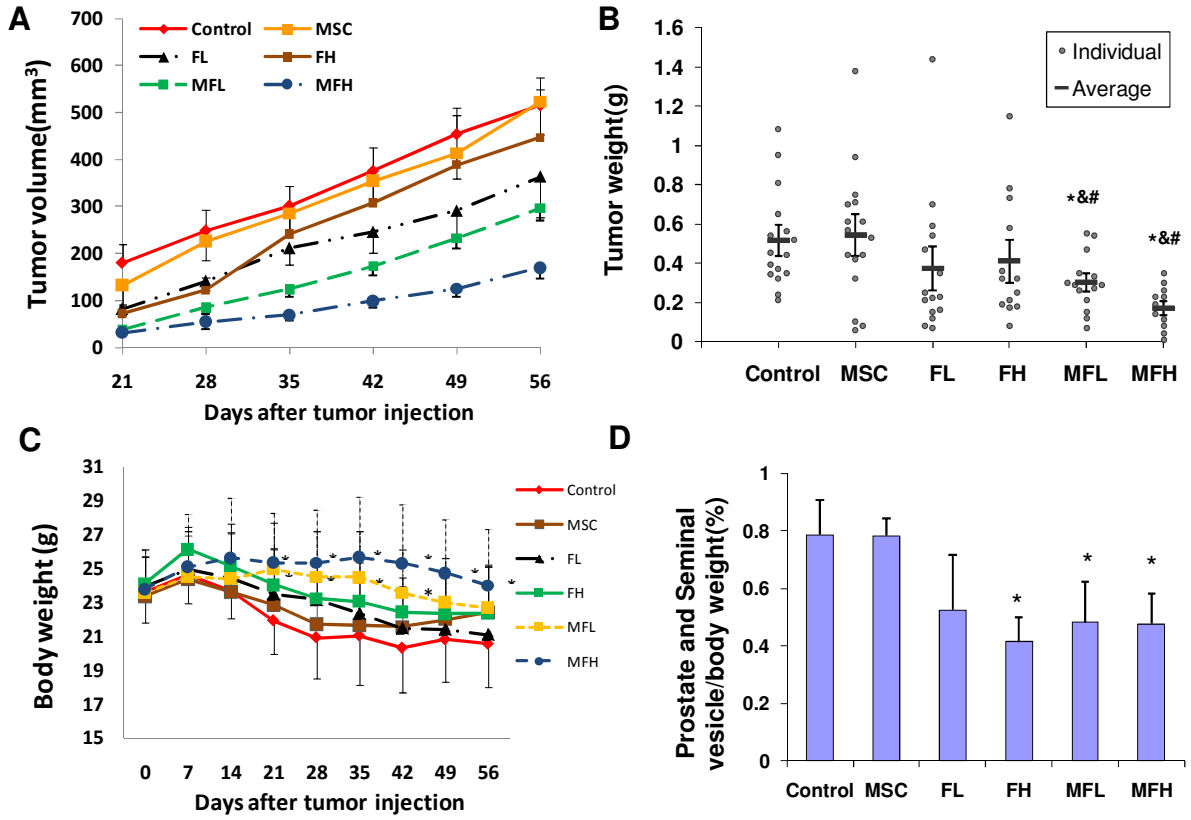


Figure 8. The effect of MSC and finasteride on growth of LNCaP xenografts in nude mice. A. Tumor growth curves are constructed form serial measurements of tumor volume. B. Individual tumor weights were obtained after the animals were sacrificed. *, P<0.05 compared with Control. &, P<0.01 compared with MSC. #, P<0.01 compared with FL or FH. C. Animal body weight during the course of the experiment. D. Prostate and seminal vesicle weights were normalized by body weights. *, P<0.05 from Control.

phosphate-buffered saline and administered by intraperitoneal injection. Animals were observed daily, and tumor measurements were taken twice weekly. Tumor volumes were calculated by the following formula: length x width x height x 0.5236. Little change in tumor take rate was observed among different groups (Table 1). Tumor growth was monitored for 8 weeks. As shown in Figure 8A, MSC treatment has very little impact on tumor growth, if any. Finasteride at the 5 mg/kg dose slowed down tumor growth, but the difference was not statistically significant (p>0.05). Interestingly, the higher dose of finasteride (50 mg/kg) appeared to be less effective than the lower dose in inhibiting tumor growth. In contrast, tumors in the combination groups grew at much reduced rates, and the differences are statistically significant when compared with the Control or the respective single treatment groups (P<0.05). The animals were sacrificed after 8 weeks, and the tumor weights correlate very closely with the tumor volume data (Figure 8B). No adverse effects were observed in animals receiving treatments. In fact, animals receiving the combination treatments had less weight loss than animals in the Control group (Figure 8C), possibly due to reduced tumor burden in these animals. As expected, the weight of the prostate and the seminal vesicle was

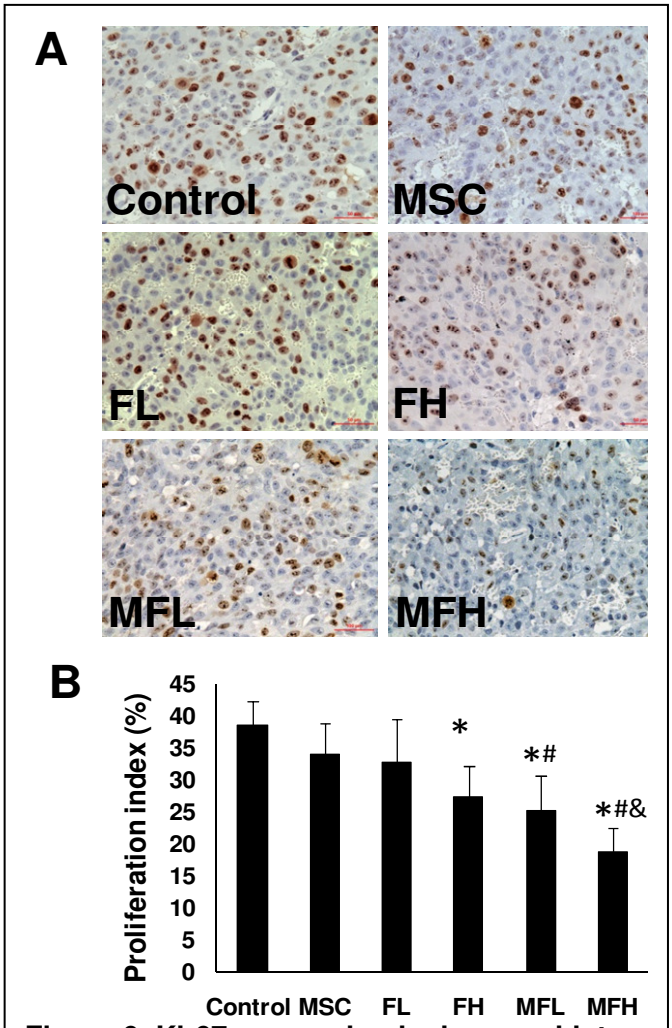


Figure 9. Ki-67 expression by immunohisto-chemistry. A, representative slides from different treatment groups. B, quantitative analysis of Ki-67 staining. *, P<0.05 vs Control. #, P<0.05 vs MSC. &, P,0.05 vs finasteride.

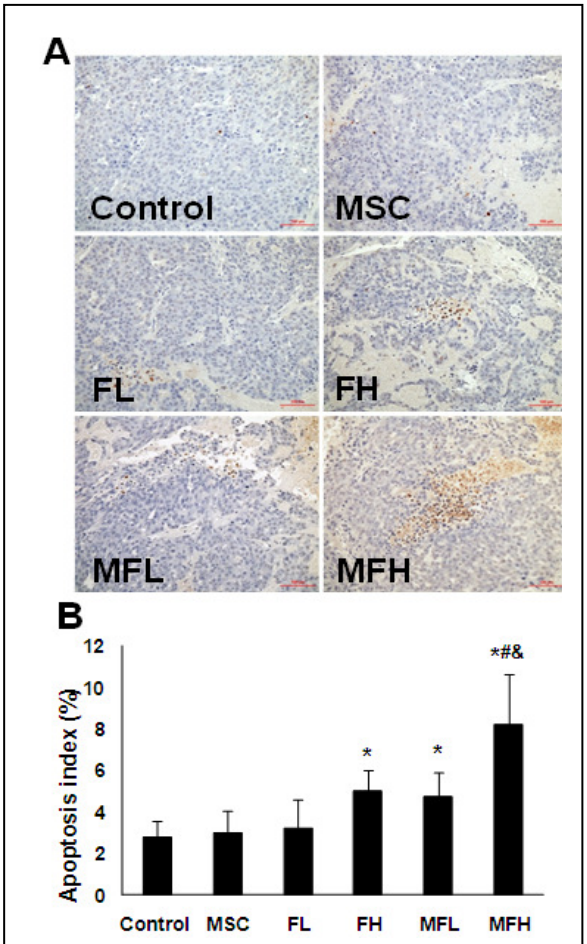


Figure 10. TUNEL assay of LNCaP xenografts. A, representative slides from different treatment groups. B. quantitative analysis of TUNEL staining. *, P<0.05 vs Control. #, P<0.05 vs MSC. &, P,0.05 vs finasteride.

reduced by finasteride but not MSC (Figure 8D), suggesting that MSC has no adverse effect on normal prostate. In summary, the tumor growth results showed that at the doses tested, neither MSC nor finasteride alone has a significant impact on inhibiting tumor growth. However, synergistic growth inhibitions were observed with two different combinations of MSC and finasteride.

Tumor cell proliferation was determined by immunohistochemistry using an anti-Ki-67 antibody. As shown in Figure 9, neither selenium nor finasteride at the lower dose affect cell proliferation. Despite have no effect on tumor volume, finasteride at the 50 mg/kg dose reduced cell proliferation by ~30%. Samples from the MFH group showed dramatic decreases in cell proliferation. In addition, apoptosis was analyzed by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Once again, the combination of MSC and finasteride at the higher dose induced apoptosis more effectively than either alone (Figure 10).

Task 4. Determine whether selenium affects the transactivation activity of FOXO1

In order to study the effect of MSA on the transcriptional activity of FOXO1, we transiently transfected LNCaP and LAPC-4 cells with a luciferase reporter construct, p3xIRS-luc. This construct has 3 tandem repeats of a FOXO1 binding element, the insulin-responsive sequence (IRS), inserted upstream of the minimal thymidine kinase promoter (6). It is widely used as an indicator of the transcriptional activity of FOXO1. Following transfection, cells were trypsinized, re-plated and were allowed to attach overnight before the addition of 10 μM MSA to the culture medium. At 6 or 16 hr, the cells were lyzed with 1X Passive Lysis Buffer (Promega). The luciferase activity was determined by using a luciferase kit from Promega, and was normalized to the protein concentration in the cell lysate. As shown in Figure 11, the transcriptional activity of FOXO1 was induced by 2-fold after 6 hr of MSA treatment. A greater than 5-fold induction was observed after 16 hr.

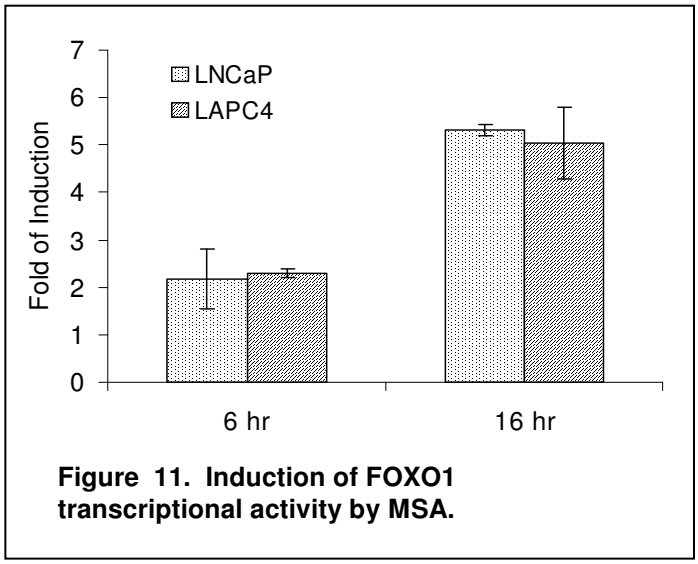


Figure 11. Induction of FOXO1 transcriptional activity by MSA.

In the proposal, we alluded to the possibility that finasteride may potentiate induction of FOXO1 activity by MSA since the interaction between AR and FOXO1 is androgen-dependent. Therefore, in the presence of finasteride and MSA, the DHT-AR complex should reduced to a greater extent because finasteride decreases the formation of DHT, while MSA depresses the abundance of AR protein. To study the effect of finasteride/MSA on FOXO1 transcriptional activity, we transfected LNCaP cells with the p3xIRS construct as described above. Cells were treated with 10 nM finasteride, or 5 μM MSA, or both. As shown in Figure 12, finasteride alone had no effect on FOXO1 activity, MSA alone produced only a small increase, due to the low concentration used in this experiment. However, the combination of finasteride/MSA resulted in an

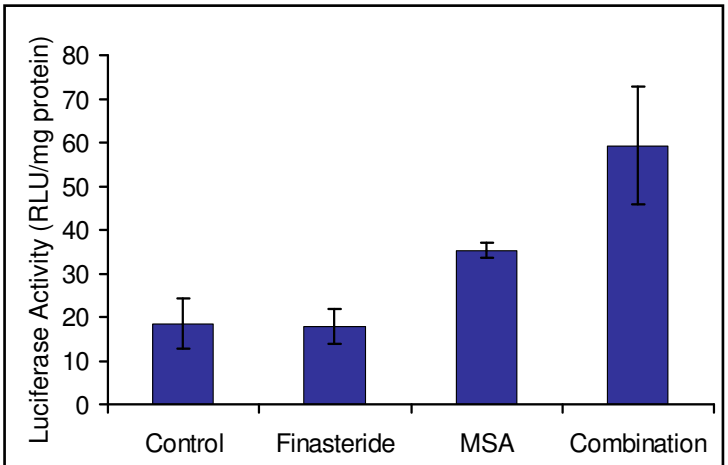


Figure 12. Induction of FOXO1 transcriptional activity by the finasteride and MSA combination.

exaggerated increase of FOXO1 transcriptional activity, suggesting a cooperative interaction between the two drugs.

Task 5. Evaluate the effect of FOXO1 knockdown on the growth inhibitory action of selenium

To further establish the role of FOXO1 in MSA-induced apoptosis, we employed the RNA interference (RNAi) technique to knockdown the expression of FOXO1. A small interfering RNA (siRNA) designed against FOXO1 (siFOXO1) was obtained from Invitrogen and transiently transfected into LNCaP cells using Lipofectamine 2000. A scrambled oligonucleotide was used as the negative control. At 48 hr post transfection, 10 μM MSA was added to the culture medium and the cells were treated for an additional 24 hr. RNA was prepared from the cells and qRT-PCR was performed to determine the efficiency of gene silencing. As shown in Fig. 13, siFOXO1 was able to decrease the baseline expression of FOXO1 by approximately 50%. Consistent with our previous finding, a 2-fold induction of FOXO1 was observed when the cells were treated with MSA for 24 hr (comparing columns 1 and 3). This induction was abolished when siFOXO1 was present (comparing columns 3 and 4).

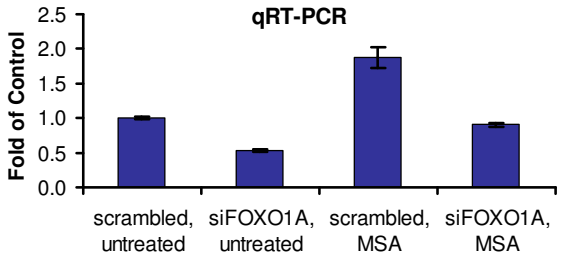


Figure. 13. Quantitation of FOXO1 mRNA in cells transfected with FOXO1 siRNA and treated with or without MSA.

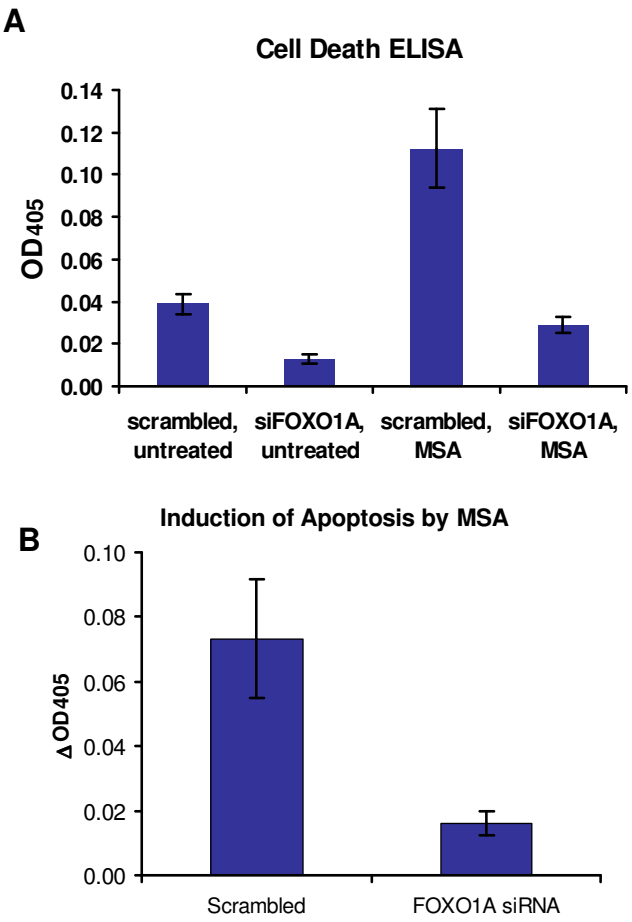


Figure. 14. A. Quantitation of apoptosis in the same samples as in Fig. 10. B. Replot of A as induction of apoptosis by MSA.

Apoptosis was also quantitated in the siRNA-transfected and MSA-treated cells by using the Cell Death Detection ELISA kit (Roche). The method is highly specific and sensitive for quantitation of apoptotic cell death. As shown

in Figure 14A, FOXO1 knockdown decreased both the baseline and MSA-induced apoptosis. The level of apoptosis correlated well with the expression level of FOXO1 (Figure 13), suggesting that FOXO1 is critical for both MSA-dependent and –independent apoptosis. More importantly, in the absence of FOXO1 siRNA, MSA increased apoptosis by 0.075 OD_{405} units (Figure 14B). However, in the presence of FOXO1 siRNA, the increase was reduced to 0.016 units (Figure 14B). Similar results were also obtained in LAPC-4 cells (data not shown).

Task 6. Determine whether AR overexpression could mitigate the modulation of FOXO1 activity by selenium

To determine whether selenium induction of FOXO1 trans-activation is mediated in part by decreasing the level of AR, we co-transfected an AR expression vector, pAR-FL, or the empty vector, pcDNA3.1, together with the FOXO1 activity reporter construct p3XIRS-luc, into LNCaP cells. Following transfection, cells were trypsinized, re-plated, and allowed to attach for 24 hr before 10 μM MSA was added to the medium. Cells were treated with MSA for 16 hr before lysed for luciferase assay. Total protein concentration was also determined to normalize the luciferase result. As shown in Figure 15A, ectopic expression of AR diminished both the baseline and MSA-induced FOXO1 trans-activation activity. This is consistent with previously published reports that AR negatively regulated FOXO1 activity (7,8). When re-plotted as induction by MSA (Figure 15B), it is obvious that MSA induction of FOXO1 activity was partially reversed in cells expressing exogenous AR. This result suggests that AR indeed plays a role in the induction of FOXO1 activity by MSA.

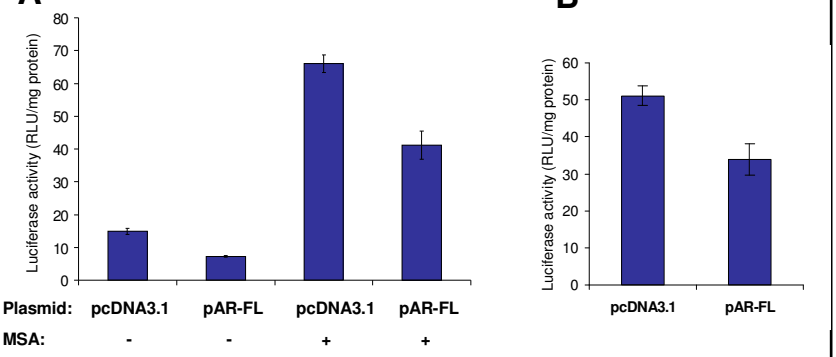


Figure 15. Ectopic expression of AR reversed the induction of FOXO1 activity by MSA. B is a re-plot of A as induction by MSA.

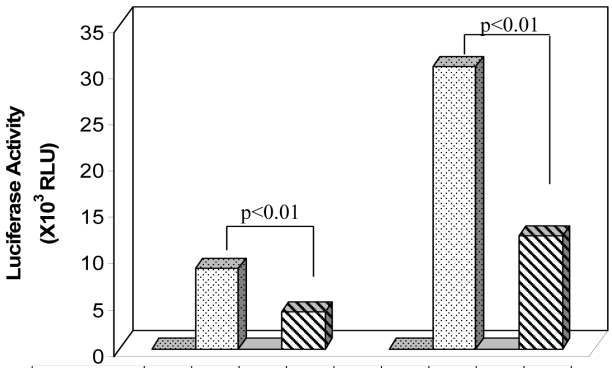
Task 7. To determine the effect of selenium on the interaction between AR and FOXO1A.

FOXO1 activation suppresses AR *trans*-activation. To examine the effect of FOXO1 activation on the transcriptional activity of AR, we transiently co-transfected LNCaP cells with a reporter construct containing 3 repeats of the androgen response element (ARE) ligated in tandem to the luciferase reporter, together with a *FOXO1* expression vector, pcDNA3-FKHR, or the empty vector. The ARE-luciferase reporter assay is commonly used to assess the *trans*-activating activity of AR. Following transfection, cells were exposed to 1 nM R1881, a synthetic androgen, for 6 or 16 hr before they were lysed for luciferase assay. As shown in Figure 16A, the AR transcriptional activity was greatly

stimulated by the addition of the ligand. In the presence of ectopically expressed FOXO1, the induction was significantly diminished (Figure 16A, comparing columns 2 and 4 for both time points). Therefore, our results confirmed published studies showing that FOXO1 activation suppresses AR signaling (9,10).

***FOXO1* induction contributes to AR suppression by MSA.** It has been found previously that MSA is a potent suppressor of AR signaling (11-13). The mechanisms involved in suppression of AR signaling by MSA include reduction in AR mRNA transcription and stability, increase in AR protein turnover, reduction in AR translocation, inhibition of coactivator recruitment, and increased corepressor recruitment to the promoters of AR-regulated genes(11-13). The result from the previous section prompted us to investigate whether *FOXO1* induction is a contributing factor for AR suppression by MSA. Once again, we employed the gene knockdown approach. LNCaP cells were co-transfected with the ARE-luciferase construct and siFOXO1, and treated with 0 or 10 µM MSA. In the presence of the scrambled oligo, MSA suppressed AR activity by approximately 70%

a. FOXO1 overexpression on AR activity



R1881	-	+	-	+		-	+	-	+
Time (hr)	6	6	6	6		16	16	16	16
FOXO1	-	-	+	+		-	-	+	+
pCDNA3.1	+	+	-	-		+	+	-	-

b. FOXO1 knockdown on suppression of AR activity by MSA

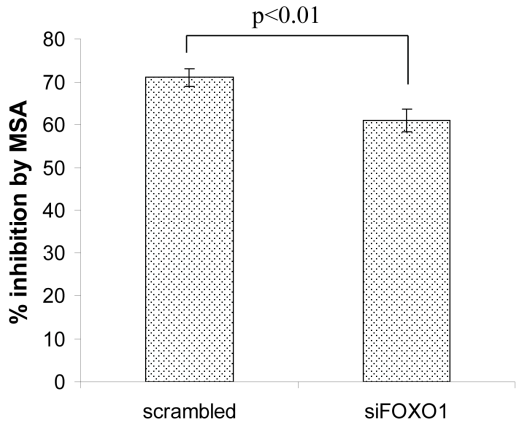


Figure 16. Induction of FOXO1 contributes to AR suppression by MSA. A. Increased expression of FOXO1 reduced the transcriptional activity of AR. LNCaP cells were co-transfected with the ARE-luciferase reporter construct and either the pcDNA3-FKHR or the pcDNA3 vector, and treated with 1 nM R1881 for the indicated times. B. FOXO1 knockdown attenuated the suppression of AR trans-activation by MSA. LNCaP cells were co-transfected with the ARE-luciferase construct and either the scrambled control or siFOXO1, and treated with 10 µM MSA for 24 hr. The luciferase reading was normalized by protein concentration. The experiment was done 3 times and the results were expressed as mean percent inhibition ± SEM.

(Figure 16B). This is in line with our previous observations. However, when *FOXO1* was silenced, the suppression was attenuated to about 60% (P<0.01). This was further confirmed when we examined the modulation of PSA expression by MSA in the presence or absence of siFOXO1 (data not shown). These results are in agreement with previous studies showing that MSA suppresses AR signaling through a multitude of mechanisms and identified *FOXO1* activation as a novel mechanism contributing to the inhibition of AR *trans*-activation by MSA.

New Task 1. Identification of FOXO1 targets modulated by selenium

As described above, we have demonstrated that selenium induces the transactivation activity of FOXO1 (Task 4), and that induction of FOXO1 is critical for apoptosis induction by selenium (Task 5). Inspired by these observations, we extended this task to identify which of the known target genes of FOXO1 is induced by selenium. Several key mediators of apoptosis, including Bim, Fas ligand, Bax, TRAIL, TRADD, and, have been shown to be regulated by FOXO members. LNCaP cells were treated with 10 μM MSA and Western blotting was carried out to analyze the expression of these FOXO1 targets. As shown in Figure 17, MSA induced expression of Bim, TRAIL, and TRADD. However, MSA has no effect on the expression of Bax and Fas ligand (data not shown). These results indicate that apoptosis induction of selenium is mediated by a subset, but not all, of the pro-apoptotic targets of FOXO.

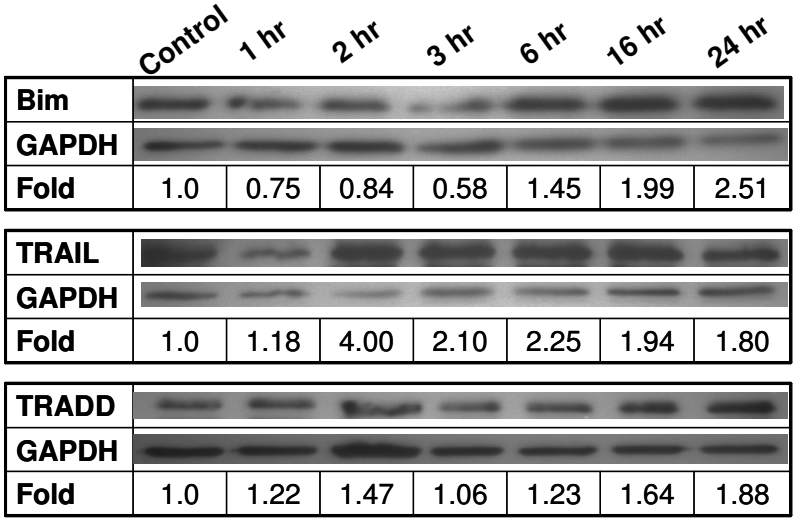


Figure. 17. Induction of FOXO1 targets by selenium.

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 (13-16).

New Task 2. To study the potential AR antagonistic activity of finasteride

Although initially designed as an inhibitor of 5α-reductase, finasteride has been found to have other biochemical effects in addition to blocking 5α-reductase. In a study by Long et al (3), 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 (14). 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 5α-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 5α-reductase gene, encoding variants of the enzyme with a low affinity for finasteride (19). 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 5α-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 18, 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

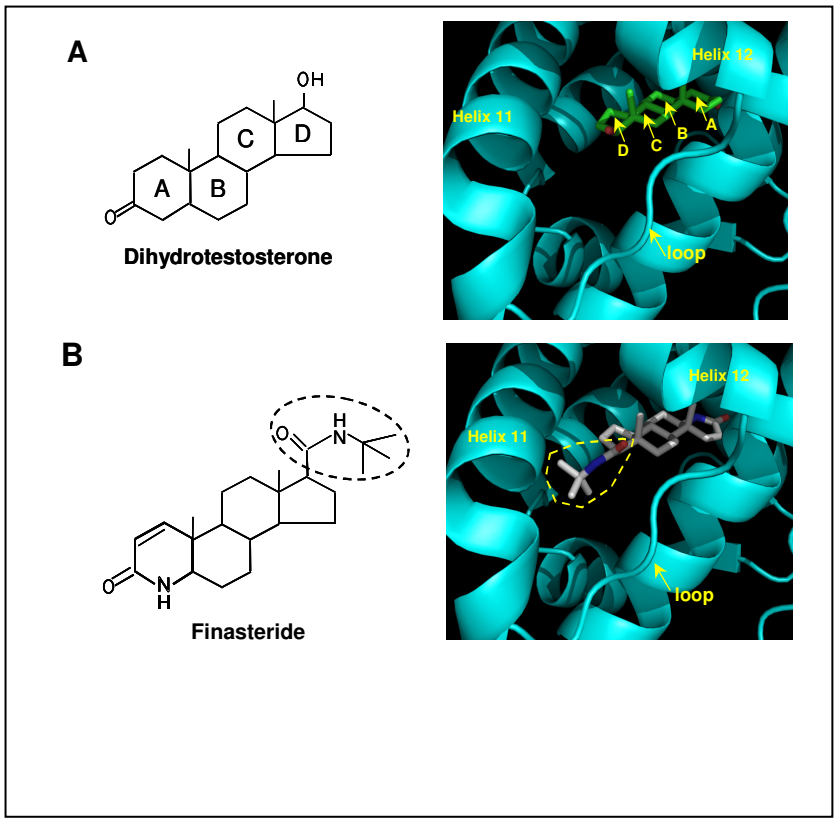


Figure 18. Docking analysis of DHT and finasteride in AR the e labeled to indicate the orientation of DHT in the AR LBD. B, finasteride. The substitution group at C17 is highlighted.

(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

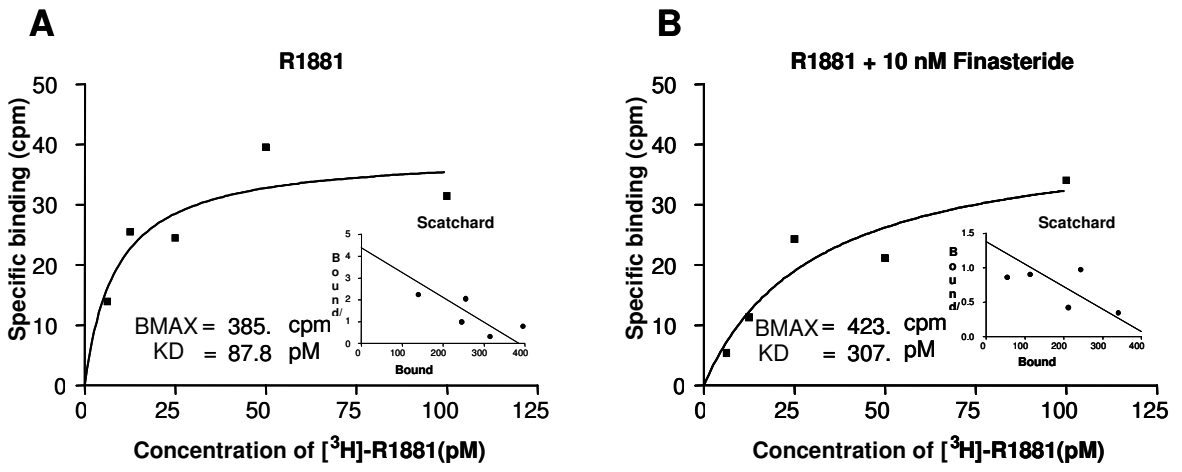


Figure. 19. Competitive ligand binding assay. A. R1881 alone. B. R1881 and 10 nM finasteride.

analyzed by Scatchard plot and is shown in Figure 19. 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.

AR antagonistic effect of finasteride

We have demonstated 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 5α-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 Figure 20, 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.

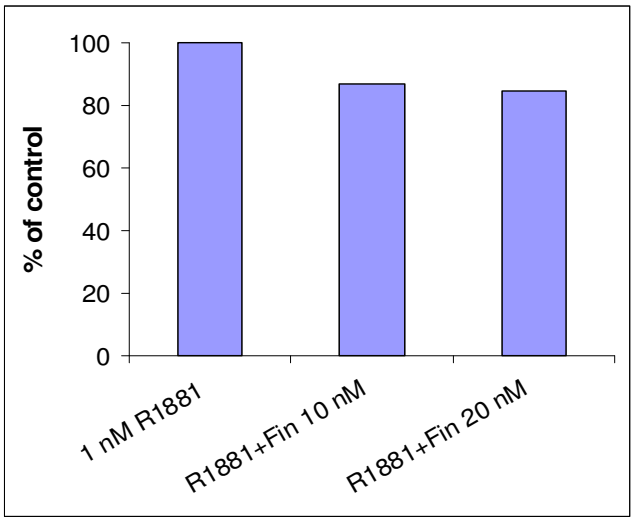


Figure 20. Potential AR antagonistic activity of finasteride.

New Task 3. Combined androgen signaling blockade by emodin and finasteride in prostate cancer chemoprevention.

This new task was inspired by an observation in the Nutrition Prevention of Cancer (NPC) trial. The NPC trial showed that the protective effect of selenium was limited to patients with baseline serum selenium in the lower 2 tertiles (16). In agreement with this observation, 78% of men in SELECT, which showed selenium supplementation did not reduce prostate cancer risk, had baseline selenium above the range that selenium provided protection in the NPC trial (<121.6 ng/ml) (17). Therefore, it is possible that individuals with high baseline selenium level will not be benefited from selenium supplementation. Therefore, we investigated the efficacy of emodin and finasteride combination in prostate cancer chemoprevention. Emodin is a phytochemical that has been shown to induce AR degradation (18). We hypothesize that the combination of emodin and finasteride synergizes on inhibiting androgen signaling and subsequently, on inhibiting tumor cells growth.

To test this hypothesis, we performed the ARE-luciferase assay in LNCaP cells treated with emodin and finasteride. As shown in Figure 21A, the activity of ARE-luciferase was stimulated by testosterone (T). Finasteride and emodin each inhibited AR activity in a dose-dependent manner. In cells that received the combination treatment, the inhibition was significantly stronger than in cells receiving single treatments. These results was confirmed when we examined the expression of prostate specific antigen (PSA), a well-known target of AR, by real-time reverse-transcription polymerase chain reaction (qRT-PCR) (Figure 21B) and Western blotting (Figure 21C). Collectively, these results suggest a synergy between emodin and finasteride in suppressing androgen signaling in prostate cancer cells.

We next examined the efficacy of emodin and finasteride in growth arrest in LNCaP cells. Cells were treated with various concentrations of emodin and finasteride for 48 hr and cell proliferation was measured by the BrdU incorporation assay. Figure 22A shows that finasteride (1 μM) had a modest effect in inhibiting cell proliferation, whereas the inhibitory effect of emodin was dose-dependent. In all the doses tested, the combination with finasteride significant enhanced the efficacy of emodin. Apoptosis induction, which was measured by using the Cell Death Detection ELISA kit (Roche) and Western blotting for PARP, showed similar results (Figure 22, B&C).

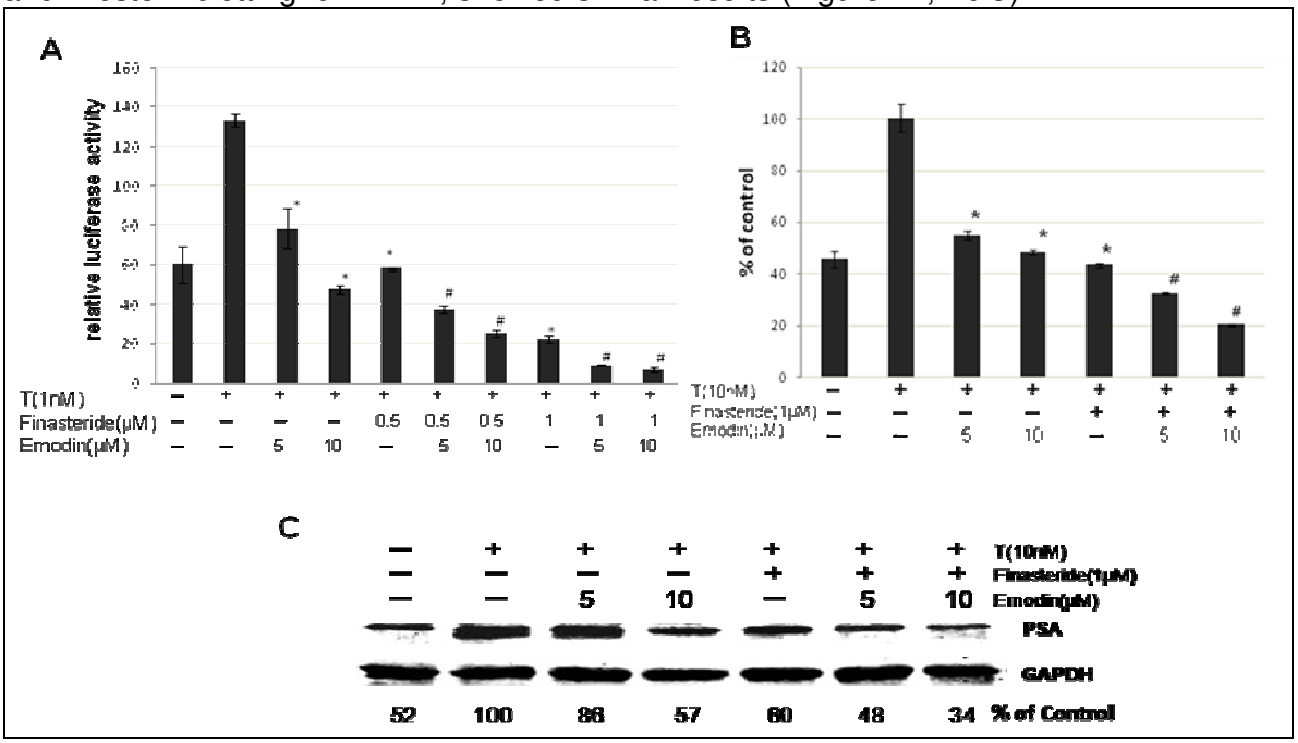


Figure 21. Suppression of androgen signaling by emodin and finasteride. A. ARE-luciferase assay. B. qRT-PCR analysis of PSA expression. C. Western blotting analysis of PSA expression. The intensity of the PSA band was normalized by that of the GAPDH. The data presented in A and B are mean ± SEM. *, statistically significant from the T-stimulated, untreated control (P<0.01); #, statistically significant from the single treatments (P<0.01).

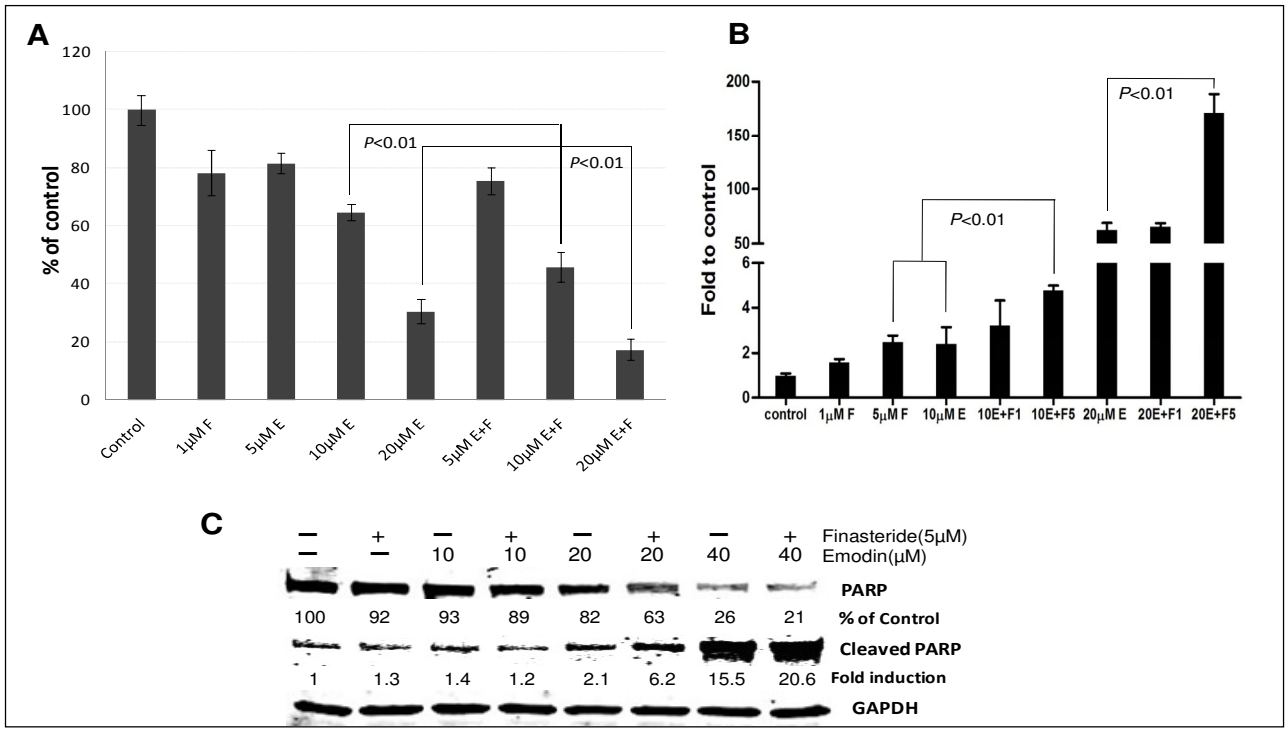


Figure 22. Effect of emodin and finasteride on growth inhibition in LNCaP cells. A. BrdU incorporation assay. B. Apoptosis assay by the Cell Death ELISA assay. C. Western blotting of PARP cleavage.

C. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that combined androgen signaling blockade by simultaneously targeting the androgen receptor and 5α-reductase is a valid and effective strategy in prostate cancer prevention.

- Consistent with recent clinical and preclinical findings, the animal experiment demonstrated that selenium compound by itself is not an effective chemopreventive agent in prostate cancer. However, MSC synergized with 5α-reductase inhibitor and effectively reduced the growth rate of tumor xenografts. This suggests a new direction for selenium compounds in prostate cancer prevention.

- Confirmed that FOXO1 and AR pathways counteract the action of each other. the action in prostate cancer cells. Demonstrated MSA modulates the balance between AR and FOXO signaling pathways.

- 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, Yue Wu, Barbara Malewicz, Junxuan Lu, Song Li, James Marshall, Clement Ip, and Yan Dong. Augmented Suppression of Androgen Receptor Signaling by a Combination of α-Tocopheryl Succinate and Methylseleninic Acid. Cancer, *107(12): 2942-2948*.

Haitao Zhang, Jian Fang, Dian Yao, Yue Wu, Clement Ip, and Yan Dong. (2010) Activation of FOXO1 is critical for the anticancer effect of selenium in prostate cancer cells. The Prostate, in press. Published on line March 23, 2010.

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

Presentation

1. Roswell Park Cancer Institute Chemotherapy/Chemoprevention Research Round, May 17, 2006, invited speech, " A combination strategy for prostate cancer chemoprevention".

2. Jilin University, Changchun, Jilin, China, August 9, 2006, invited speech, "Delinating the molecular mechanisms of prostate cancer chemoprevention by selenium".

3. Selenium 2006, Madison, WI, July 25-28, poster presentation, "A combination strategy for prostate cancer chemoprevention based on selenium suppression of androgen signaling".

4. AACR Centennial Meeting, Los Angeles, CA, April 15-18, poster presentation, "Combining selenium and finasteride for prostate cancer chemoprevention".

5. 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".

6. DOD Innovative Minds in Prostate Cancer Today meeting, Atlanta, GA, Sept 5-8, poster presentation, "Combining selenium and finasteride for prostate cancer chemoprevention".

7. Tulane University, New Orleans, Louisiana, Sept 11, 2007, invited speech, "Translational research of selenium and finasteride in prostate cancer prevention".

8. Haitao Zhang: Translational study on selenium and finasteride in prostate cancer prevention. Invited seminar. Department of Medicine, Tulane University School of Medicine. May 16, 2008.

9. Haitao Zhang: Selenium and prostate cancer: new frontiers. Invited seminar.Department of Structure and Cellular Biology, Tulane University School of Medicine. August 20, 2008.

10. Haitao Zhang: Targeting androgen signaling axis for prostate cancer intervention. Invited seminar. Jilin University School of Medicine, Changchun, Jilin Province, China. July 27, 2009.

11. Haitao Zhang: New concepts of selenium in prostate cancer intervention. Invited oral presentation. To be given in World Cancer Congress2010, Singapore, June 22 - 25

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

The results from the current study demonstrated a synergism between 5α-reductase inhibition and AR down-regulation in inhibiting the growth of prostate cancer cells both *in vitro* and *in vivo*. This finding has significant clinical implications. Since the induction of PSA screening, the majority of the prostate cancers diagnosed are asymptomatic, early-stage, small volume diseases. Current treatment options, including surgery and radiation therapy, are associated with serious quality-of-life complications. Our study suggests that the combination of finasteride and MSA could be used to prevent the clonal expansion of small-volume, low-grade prostate cancer cells, providing a novel disease management strategy.

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.

The findings presented above demonstrated that MSA activates FOXO1 signaling pathway. FOXO1 plays a critical role in mediating the apoptotic activity of MSA, and also contributes to the suppression of androgen signaling by MSA. This study enhances our understanding of the molecular mechanisms of the anticancer activity of MSA, which will be critical for designing future prostate cancer intervention studies with MSA.

The current study provides evidence that finasteride, in addition to its ability to inhibit 5α-reductase, has AR antagonistic activity. It has been shown 35% of alleles in the US population carry mutations in the type II 5α-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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Augmented Suppression of Androgen Receptor Signaling by a Combination of α-Tocopheryl Succinate and Methylseleninic Acid

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Supported by a grant K01 CA114252 from the National Cancer Institute (to Y.D.); DOD New Investigator grants W81XWH-04-1-0009 (to Y.D.) and grant W81XWH-05-1-0598 (to H.Z.); grant 62-2198-01 from the Roswell Park Alliance Foundation (to C.I.); grant CA95642 from the National Cancer Institute (to J.L.); shared resources of the Roswell Park Cancer Center Support grant P30 CA16056 from the National Cancer Institute.

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Received May 10, 2006; revision received August 23, 2006; accepted September 29, 2006.

BACKGROUND. Previous reports showed that α-tocopheryl succinate (αTS) and methylseleninic acid (MSA) independently reduce the abundance of androgen receptor (AR) in prostate cancer cells. The response to MSA happens quickly, whereas the response to αTS takes much longer. The present study was designed to investigate whether a combination of αTS and MSA would produce an additive or a greater than additive effect in suppressing AR level, AR transactivation, and prostate-specific antigen (PSA).

METHODS. LNCaP cells were treated with αTS alone for 31 hours, MSA alone for 3 hours, or αTS first for 28 hours and αTS/MSA together for the last 3 hours. AR and PSA mRNA levels were quantitated by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). AR transactivation was determined by the ARE-luciferase reporter assay. Both cellular and secretory PSA was also measured by the enzyme-linked immunosorbent assay (ELISA) method.

RESULTS. Different doses of αTS were evaluated in combination with MSA. Some striking results are highlighted below for αTS alone, MSA alone, or αTS/MSA (presented in that order). AR mRNA level was depressed by 0%, 20%, or 60%, respectively; AR transactivation was inhibited by 35%, 10%, or 60%, respectively; whereas the PSA mRNA level was decreased by 40%, 60%, or 90%, respectively. Interestingly, secretory PSA was consistently reduced to a greater extent than cellular PSA.

CONCLUSIONS. A combination of αTS/MSA produced a greater than additive effect in suppressing AR signaling compared with the single agent. Decreased AR abundance is a major factor, but not necessarily the sole factor, in diminishing the transcriptional activity of AR by αTS or MSA. ***Cancer*** **2006;107:2942–8.** © *2006 American Cancer Society.*

KEYWORDS: androgen receptor, prostate-specific antigen, α-tocopheryl succinate, methylseleninic acid.

There is extensive documentation that androgen is required for the development of prostate cancer in humans.[1] Testosterone and dihydrotestosterone (DHT) are the 2 key androgens in men. Because DHT binds to the androgen receptor (AR) with a greater affinity than does testosterone, it is the more potent androgen in a biologic sense. Steroid 5α-reductase is the enzyme responsible for catalyzing the irreversible conversion of testosterone to DHT.[2] Many synthetic inhibitors of 5α-reductase have been developed, although only 1, finasteride, was successfully shown to reduce the prevalence of prostate cancer by 25% in low-risk men.[3] In view of the modest chemopreventive effect of finasteride, additional research aimed at identifying nontoxic agents capable of disrupting androgen signaling beyond the 5α-reductase step would be highly desirable.

DOI 10.1002/cncr.22345
Published online 21 November 2006 in Wiley InterScience (www.interscience.wiley.com).

Recently, α-tocopheryl succinate (αTS) and methylseleninic acid (MSA) have been reported independently to reduce the expression of AR transcript and protein in human LNCaP prostate cancer cells.[4–7] The kinetics of AR depression is very different under these 2 treatments. The effect of MSA is acute, whereas the effect of αTS is delayed. These observations imply that MSA and αTS may have different mechanisms in down-regulating the AR level. In the present study, we carried out a series of experiments to investigate whether αTS and MSA in combination would produce an augmented effect. We analyzed AR message and protein levels as well as AR transactivating activity by a reporter gene assay. For a prototypical AR target, we measured prostate-specific antigen (PSA) changes at the message and protein levels. Both cellular PSA and secretory PSA were evaluated after αTS/MSA treatment.

The significance of this research will be discussed in relation to a population-based prostate cancer chemoprevention trial.

MATERIALS AND METHODS

Cell Culture and Treatment

The human LNCaP prostate cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). The cells, with a passage number of 40 to 45, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM of glutamine. Treatment with αTS or MSA began at 72 hours or 100 hours after seeding, respectively, when the cultures reach a confluency of 60% to 80%. Cells were harvested after 31 hours if treated with αTS alone, or after 3 hours if treated with MSA alone. If cells were treated with the combination of αTS and MSA, they were exposed first to αTS for 28 hours, then to MSA for the last 3 hours (with αTS still present in the medium) before harvesting. These timepoints were chosen based on prior studies of the duration required to achieve a suppression of AR signaling by MSA or αTS as a single agent.[4–7] A significant down-regulation of AR protein level is readily detectable between 3 and 6 hours of MSA treatment. In contrast, an exposure time of 24 to 48 hours to αTS is normally necessary to suppress AR signaling. The experiment was repeated 3 times and the RNA and cell lysates collected and subjected to real-time reverse-transcriptase polymerase chain reaction (RT-PCR) or Western analysis, respectively. αTS was purchased from Sigma (St. Louis, MO). MSA was synthesized as described previously.[8]

Real-Time RT-PCR

The PCR primers and Taqman probes for AR, PSA, and α-actin (a housekeeping gene) were Assays on-Demand products from Applied Biosystems (Foster City, CA). The PCR conditions were as follows: an initial incubation at 50°C for 2 minutes, then a denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantitation of gene expression was done using the comparative CT ($\Delta\Delta C_T$) method.[9] Details of the procedure were described in our previous publication.[4] Each real-time RT-PCR experiment was done in triplicate and the mean C_T value was used for data analysis. The final result is presented as the mean of 3 separate experiments ± standard error.

Western Blot Analysis

The following monoclonal antibodies were used: anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA), anti-AR (BD Biosciences, San Diego, CA), and anti-PSA (Lab Vision, Fremont, CA). Immunoreactive bands were quantified by volume densitometry with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and normalized to GAPDH. Densitometry calculates the volume and density of a given immunoreactive band on the film and provides a semiquantitative analysis of the Western results. Three independent experiments were performed and the result of a representative experiment is presented.

Reporter Gene Assay

The ARE-luciferase reporter plasmid, containing 2 repeats of the ARE region ligated in tandem to the luciferase reporter,[10] was transiently transfected into cells at a concentration of 9 μg per 10-cm culture dish. The transfection was carried out using the Lipofectamine and Plus reagents (Invitrogen, Carlsbad, CA) per instruction of the manufacturer. After incubating with the transfection mixture for 3 hours, the cells were trypsinized, resuspended in a medium containing charcoal-stripped serum and 10 nM DHT (Sigma), and plated in triplicate onto 6-well plates. Cells were allowed to recover for 24 hours before treatment with αTS and/or MSA. At the end of the treatment, cells were lysed with the reporter lysis buffer (Promega, Madison WI), and the luciferase activity was assayed using the Luciferase Assay System (Promega). Protein concentration in the cell extract was determined by the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) per instruction of the manufacturer. Luciferase activities were normalized to the protein concentration of the sample. The

transfection experiments were repeated 3 times. The treatment-to-control ratios were calculated and averaged for the triplicate measurements within each individual experiment. The result is presented as the mean of 3 separate experiments ± standard error.

PSA Measurement by ELISA

The cell cultures were prepared slightly differently for PSA measurement by ELISA, as reported previously.[5] At 72 hours after seeding, cells were rinsed 3 times with phosphate-buffered saline (PBS) to reduce the carryover of residual conditioned medium before treatment. Based on our experience, the concentration of the secreted PSA in the conditioned medium could reach 50–100 ng/mL after this 72 hours of incubation. The washing brought the level down to within the limit of detection. At the end of the treatment, conditioned media were collected and the detached cells were removed by centrifugation. Cell lysate was prepared in PBS by 3 cycles of freezing and thawing, followed by 15 seconds of sonication. PSA in conditioned medium and cell lysate was measured by using the MAGIWEL PSA ELISA system from United Biotech (Mountain View, CA). A pretest with all the samples as a single cell was performed. Based on the signals obtained, dilutions of the samples were made to ensure that all the samples were measured in the linear range of the assay (up to 30 ng/mL of the PSA standard provided by the manufacturer). The dilutions were made in duplicate and the ELISA activities were normalized to the protein concentration of the sample.

Statistical Analysis

The Student 2-tailed *t*-test was used to determine significant differences between treatment and control values and $P < .05$ was considered statistically significant.

RESULTS

Dose Response of AR Down-Regulation by αTS or MSA

In order to select the appropriate dose of αTS and MSA to use in the combination, it was important to first find out the sensitivity of AR to each agent. We tested αTS at 20, 30, or 40 μM and MSA at 2.5, 5, or 10 μM. As noted in Materials and Methods, cells were harvested at 31 hours after αTS treatment and at 3 hours after MSA treatment. AR level was quantified by real-time RT-PCR. αTS reduced AR expression by 0%, ~10%, or ~60% at concentrations of 20, 30, or 40 μM, respectively (data not shown). We repeated this experiment a number of times and confirmed that the AR dose response to αTS was apparently very steep between 30 to 40 μM. Conversely, MSA reduced AR expression by ~20%, ~40%, or ~60% at concentrations of 2.5, 5, or 10 μM, respectively (data not shown). Thus, the AR dose response to MSA was linear in this range. The above information was helpful in deciding the combination dosage. In order to leave room to detect an additive or greater than additive effect, we clearly did not want to use a dose of MSA that by itself would have produced a substantial reduction of AR. On this basis, we chose either 20 μM αTS/2.5 μM MSA or 40 μM αTS/2.5 μM MSA for the combination experiments.

Combined Effect of αTS/MSA Treatment on AR Depression

Cells were treated with αTS first for 28 hours, followed by αTS and MSA for 3 more hours before harvesting. We did not change the medium at the time MSA was added. The single-agent culture was treated with either αTS alone for 31 hours or MSA alone for the last 3 hours. The quantitative RT-PCR AR level from the 31-hour untreated control culture was set at 100%. The AR data from the 3 treated cultures (αTS alone, MSA alone, αTS + MSA) are expressed as percent of control as shown in Figure 1A. αTS at 20 μM had no effect on AR mRNA level, whereas MSA at 2.5 μM reduced AR to ~80% of control. A combination of 20 μM αTS/2.5 μM MSA, however, decreased AR to 37% of control. When αTS was raised to 40 μM, there was a robust reduction of AR down to ~30% of control. Combining this dose of αTS with MSA further depressed the AR level to 10% of control. The Western blot data of the second experiment are shown in Figure 1B. The protein results are also expressed as percent of control. It can be seen that the Western blot data are consistent with the mRNA data with respect to the magnitude and pattern of change. The Western analysis was not performed with the first combination because no decrease in AR protein level was detected with either 20 μM αTS or 2.5 μM MSA (data not shown).

Combined Effect of αTS/MSA Treatment on AR Transactivating Activity Inhibition

A low abundance of AR is expected to diminish AR transactivation. The ARE-luciferase reporter assay is commonly used to assess AR transactivating activity. Figure 2 shows the results of the effects of αTS/MSA with this assay. The data are also expressed as percent of untreated control. αTS alone at 40 μM decreased AR activity to ~65% of control, whereas MSA alone at 2.5 μM produced only a 10% inhibition at best. The combination, conversely, depressed AR activity to ~40% of control. The results are congruent

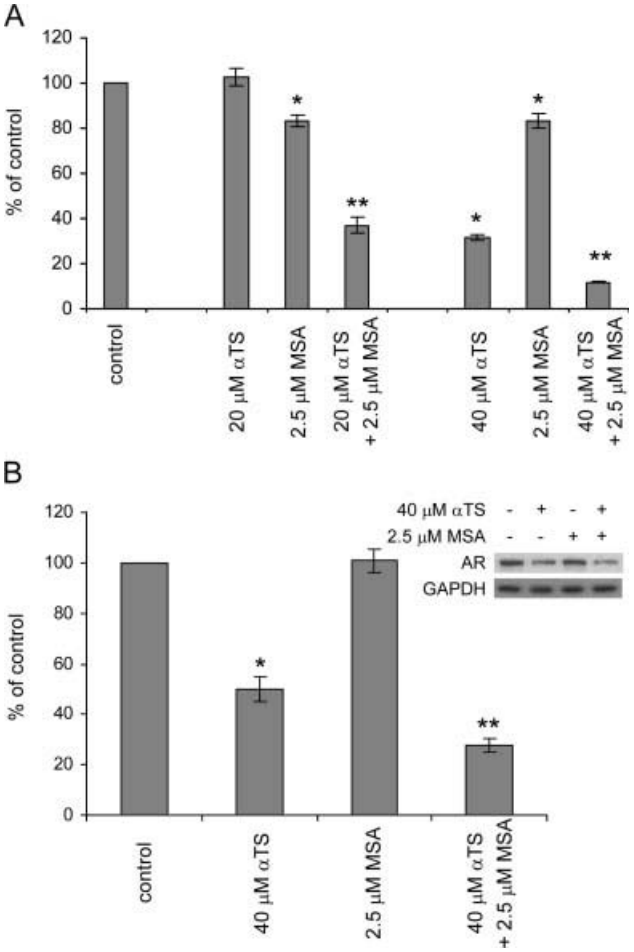

FIGURE 1. Effect of α-tocopheryl succinate (αTS) and/or methylseleninic acid (MSA) on androgen receptor (AR) expression. (A) Changes in AR mRNA level as determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). (B) Western blot data of changes in AR protein level. The values represent mean ± SE (n = 3). *Statistically different from the untreated control, $P < .05$. **Statistically different from either of the single-agent treatments, $P < .05$.

with the interpretation that the inhibition of AR transactivation was in part accounted for by the reduction of AR protein.

Combined Effect of αTS/MSA Treatment on PSA Depression

PSA is a well-accepted AR-regulated target. A decrease in AR transactivation is expected to depress PSA production. PSA expression was quantified by real-time RT-PCR (Fig. 3A) and Western blot (Fig. 3B) analyses. αTS at 20 μM or MSA at 2.5 μM reduced PSA mRNA to ~60% and 40% of control, respectively. Combining αTS and MSA at these concentrations knocked down PSA expression to ~10% of control. We also used a higher concentration of αTS at 40 μM, because the AR dose-response curve was very steep between 20 and 40 μM of αTS. At 40 μM αTS, PSA mRNA was depressed to less than 10% of control. The combination of 40 μM αTS and 2.5 μM MSA almost completely blocked the expression of PSA mRNA. The Western blot PSA data (Fig. 3B) tracked closely with the mRNA data.

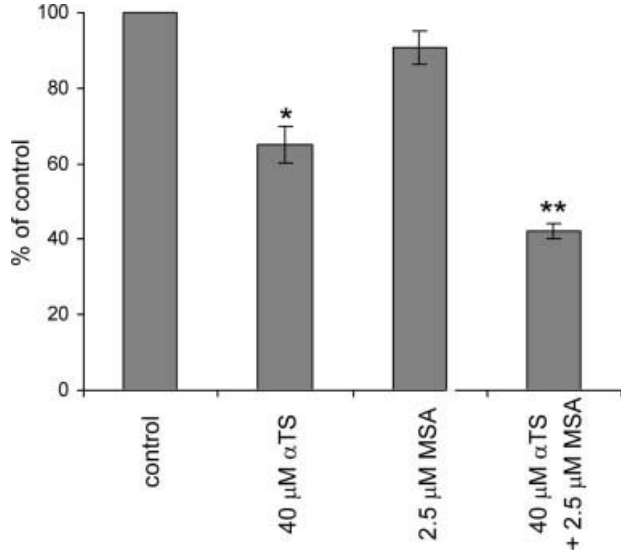

FIGURE 2. Effect of α-tocopheryl succinate (αTS) and/or methylseleninic acid (MSA) on ARE-luciferase activity. The values represent mean ± SE (n = 3). *Statistically different from the untreated control, $P < .05$. **Statistically different from either of the single-agent treatments, $P < .05$.

Differential Sensitivity of Cellular Versus Secretory PSA to αTS/MSA Inhibition

PSA produced by cultured cells is secreted into the medium. In order to compare the sensitivity of cellular and secretory PSA to αTS/MSA inhibition, we used an ELISA method to measure PSA in both fractions. We studied 2 combinations: 20 μM αTS/2.5 μM MSA, or 40 μM αTS/2.5 μM MSA. The results, which are expressed as percent of untreated control, are shown in Figure 4. It was no surprise to find that the 40 μM αTS/2.5 μM MSA combination was more potent than the 20 μM αTS/2.5 μM MSA combination in inhibiting PSA. Thus, qualitatively the ELISA method gave the same kind of results as the qRT-PCR method. An interesting observation from this experiment was that in every treatment condition except MSA alone, secretory PSA was suppressed to a greater degree than cellular PSA.

DISCUSSION

In this study we found that a combination of 20 μM αTS and 2.5 μM MSA markedly depressed AR expres-

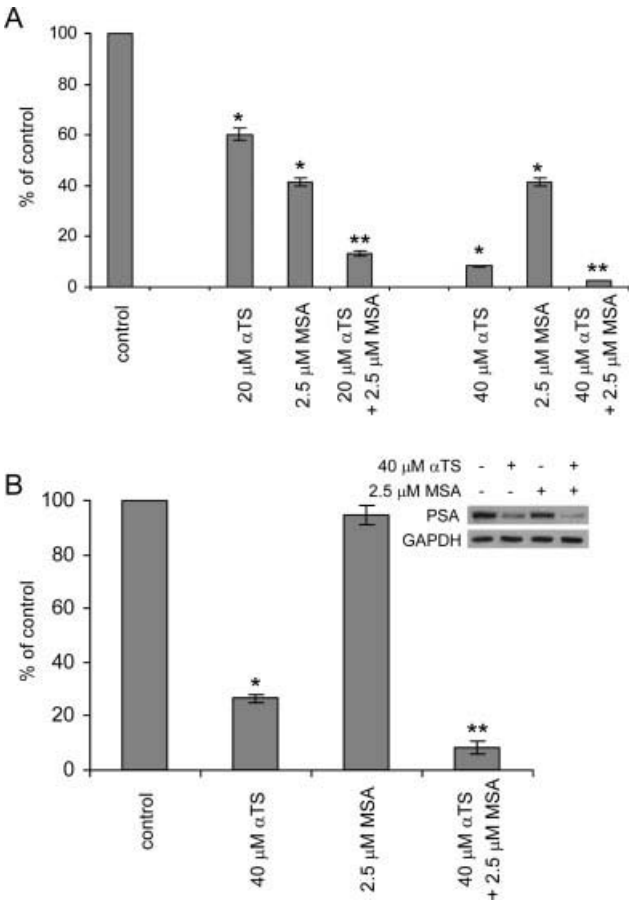

FIGURE 3. Effect of α-tocopheryl succinate (αTS) and/or methylseleninic acid (MSA) on prostate-specific antigen (PSA) expression. (A) Changes in PSA mRNA level as determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). (B) Western blot data of changes in cellular PSA protein level. The values represent mean ± SE (n = 3). *Statistically different from the untreated control, $P < .05$. **Statistically different from either of the single-agent treatments, $P < .05$.

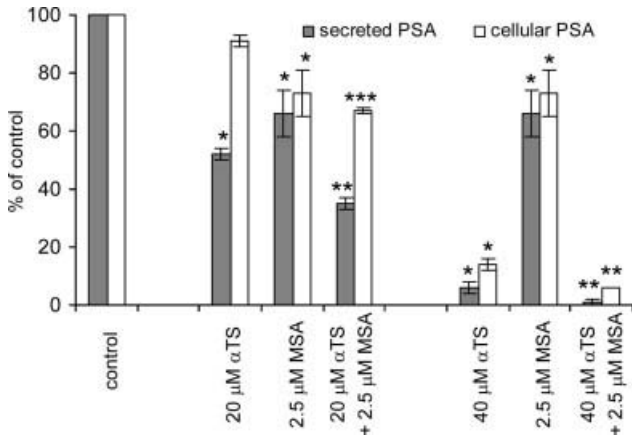

FIGURE 4. Effect of α-tocopheryl succinate (αTS) and/or methylseleninic acid (MSA) on cellular and secretory prostate-specific antigen (PSA) as determined by enzyme-linked immunosorbent assay (ELISA). The values represent mean ± SE (n = 3). *Statistically different from the untreated control, $P < .05$. **Statistically different from either of the single-agent treatments, $P < .05$. ***Statistically different from αTS treatment only, $P < .05$.

sion to ~40% of control, although αTS or MSA by itself had minimal effects. An important question to address is whether the decrease in AR is secondary to growth inhibition by these agents. We have reported previously that neither 20 μM αTS nor 2.5 μM MSA produced any significant effect on the growth of LNCaP cells even after 48 hours of treatment.[4,11] In assessing the response of AR to αTS and MSA, the cells were treated with αTS first for 28 hours, followed by αTS and MSA for another 3 hours. Thus, it is unlikely that the down-regulation of AR under this condition is related to cytotoxicity. When the concentration of αTS was raised to 40 μM, AR level was reduced to 30% of control. This concentration of αTS would have produced ~50% growth inhibition at the time the cells were harvested for AR quantification.[11] Therefore, the AR results generated from any treatment protocol with 40 μM αTS would be more difficult to interpret. Nonetheless, the fact that we were still able to detect a greater AR suppression by 40 μM αTS/2.5 μM MSA than by 40 μM αTS alone suggests that these 2 agents may work cooperatively in modulating AR expression.

Our previous report showed that overexpression of AR considerably weakened the inhibitory effect of MSA on cell growth and proliferation as well as the expression of AR target genes in LNCaP cells.[6] The findings indicate a key role of AR down-regulation in mediating the anticancer effect of MSA in prostate cancer. The silencing of AR by siRNA has recently been demonstrated to lead to increased apoptosis,[12] further suggesting that restricting AR expression can impact cell biology. Therefore, depletion of AR could represent an alternative strategy of prostate cancer control, or, at the very least, provide a complementary approach to androgen deprivation treatment.

A careful examination of the expression levels of AR and PSA in αTS- or MSA-treated cells reveals additional information of interest. αTS at 20 μM had a minimal effect on AR expression (Fig. 1A), but reduced PSA mRNA level by as much as 40% (Fig. 3A). The same kind of discrepancy was also evident with MSA treatment. MSA at 2.5 μM decreased AR expression by no more than 20% (Fig. 1A), but depressed PSA mRNA level by 60% (Fig. 3A). The results suggest that AR suppression is a major factor, but not necessarily the sole factor, in diminishing the transcriptional activity of AR by αTS or MSA. AR sig-

naling begins with binding of DHT to the receptor and subsequent translocation to the nucleus. The activated receptor then binds to the ARE in the promoter of the target gene. Transcriptional activity is further regulated by the recruitment of coactivators or corepressors.[13] αTS and MSA may potentially modulate 1 or more of these steps (unpubl. data).

The ongoing SELECT trial is testing the efficacy of selenium and/or α-tocopheryl acetate in prostate cancer prevention.[14] αTS is a synthetic derivative of α-tocopheryl, and it is the most commonly used form of vitamin E analog in in vitro studies of cancer research. The hydroxyl group in position C6 of the chroman head is esterified to succinic acid (a dicarboxylic acid) in αTS, rendering αTS more hydrophilic than α-tocopherol. It is generally believed that αTS is taken up more efficiently by cells than α-tocopherol. More than a decade ago, Turley et al.[15] showed that αTS at a concentration of 30 μM caused growth arrest in HL-60 cells, whereas α-tocopherol and α-tocopheryl acetate did not arrest growth even at a concentration as high as 100 μM and 200 μM, respectively. These observations have since been corroborated by many investigators in different cell models, including LNCaP and PC-3 human prostate cancer cells.[16] However, the above observations were obtained after 72 hours of treatment at the longest. It is possible that α-tocopherol and α-tocopheryl acetate are taken up by cells at a much slower rate than αTS. Therefore, it remains to be determined whether α-tocopherol or α-tocopherol acetate may induce growth inhibition and suppress AR signaling after prolonged treatment.

The form of selenium used in the SELECT trial is selenomethionine.[14] As discussed previously,[17] cultured prostate cells respond poorly to selenomethionine and only when it is present at supraphysiologic levels in the medium. A plausible explanation is that prostate cells have a low capacity in metabolizing selenomethionine to methylselenol, which is believed to be the active species for the anticancer activity of selenium.[18] This process normally takes place in the liver and kidney. For this reason, MSA, an oxidized form of methylselenol, was developed by Ip et al[8] specifically for in vitro experiments. Once taken up by cells, MSA is readily reduced by glutathione and NADPH to methylselenol (which is rather unstable in itself) via a nonenzymatic reaction. The cellular and molecular responses of prostate cells to physiologic concentrations of MSA have been documented in a number of publications.[17,19–21] MSA also has excellent anticancer activity in animals. Additionally, MSA produces the same molecular biomarker changes in vivo as other seleno-amino acids. Therefore, the information obtained with MSA from cell culture studies would be relevant to the action of selenomethionine in human.

The SELECT protocol provides for the establishment of a repository for prostate biopsy tissues, blood cells, and plasmas. There will be opportunities in the future to evaluate molecular biomarkers using the banked samples. The SELECT results will not be available for a while. In the meantime, we should try to find out the effective cellular concentrations of the different vitamin E compounds and whether they could produce similar molecular alterations when the effective cellular concentrations could be reached. The clarification of these issues is important in enabling us to interpret the data from the intervention trial.

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Activation of FOXO1 Is Critical for the Anticancer Effect of Methylseleninic Acid in Prostate Cancer Cells

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BACKGROUND. Previous studies have demonstrated that physiological concentrations of methylseleninic acid (MSA) inhibits the growth of prostate cancer cells. The growth inhibitory effect could be attributed to cell cycle block and apoptosis induction. The current study was designed to investigate the involvement of forkhead box O1 (FOXO1) in the anticancer effect of MSA.

METHODS. LNCaP and LAPC-4 cells were treated with 10 μM MSA for various time points, and the expression of FOXO1 was analyzed by qRT-PCR and Western blotting. FOXO1 activity was determined by a luciferase construct containing FOXO binding sites. The *trans*-activation activity of the androgen receptor (AR) was determined by the ARE-luciferase assay. FOXO1 gene silencing was achieved by using a small interfering RNA (siRNA).

RESULTS. MSA treatment led to a rapid and robust increase of FOXO1 expression, as well as an increase of the FOXO1 transcriptional activity. Blocking FOXO1 activation by gene silencing abolished apoptosis induction by MSA, suggesting FOXO1 plays a critical role in mediating the apoptotic effect of MSA. Recent studies have shown that FOXO1 and AR antagonize the actions of each other. We examined the consequence of FOXO1 induction on AR activity. Consistent with previous reports, we found that ectopic expression of FOXO1 suppressed the transcriptional activity of AR. Furthermore, FOXO1 silencing attenuated MSA suppression of AR activity, suggesting that FOXO1 induction contributes to suppression of AR signaling by MSA.

CONCLUSIONS. In prostate cancer cells, MSA activates the FOXO1 signaling pathway. FOXO1 activation is critical for the anticancer effects of MSA. *Prostate.*

KEY WORDS: **methylseleninic acid; FOXO1; apoptosis; androgen receptor**

Abbreviations: AR, androgen receptor; ARE, androgen response element; FOXO1, forkhead box O1; MSA, methylseleninic acid; NPC, Nutritional Prevention of Cancer; PCa, prostate cancer; PSA, prostate-specific antigen; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SELECT, Selenium and Vitamin E Chemoprevention Trial; siRNA, small interfering RNA.

Grant sponsor: Department of Defense Prostate Cancer Research Program; Grant number: W81XWH-05-1-0598; Grant sponsor: American Cancer Society; Grant number: RSG-07-218-01-TBE; Grant sponsor: National Cancer Institute; Grant numbers: K01CA114252, P01CA126804, P30CA16056; Grant sponsor: Louisiana Cancer Research Consortium.

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Received 6 January 2010; Accepted 9 February 2010
DOI 10.1002/pros.21162
Published online in Wiley InterScience (www.interscience.wiley.com).

INTRODUCTION

Prostate cancer (PCa) is a significant public health problem that engenders huge medical care and human suffering costs in the United States. A number of case–control studies have demonstrated an inverse relationship between selenium status and prostate cancer risk [1–5]. One of the more important studies of selenium as a chemopreventive agent is the Nutritional Prevention of Cancer (NPC) trial initiated by Larry Clark [6,7]. The study was a randomized, double-blind, placebo-controlled trial involving 1,312 patients (mostly men) who were recruited initially because of a history of basal cell or squamous cell carcinoma of the skin. Individuals in the treatment arm were given 200 μg selenized yeast per day for a mean of 4.5 years. After a total follow-up of 8,271 person-years, selenium treatment did not decrease the recurrence of these non-melanoma skin cancers. However, patients receiving the supplement showed a much lower risk of developing total (HR = 0.75) or prostate cancer (HR = 0.48) [6,7].

Encouraged by the prostate cancer results of the NPC trial, the National Cancer Institute launched the Selenium and Vitamin E Cancer Prevention Trial (SELECT) in 2001. An interim data analysis after a median follow-up of 5.46 years suggested selenium, either alone or in combination with vitamin E, did not lower prostate cancer risk in this study population [8]. The trial was halted in October 2008, but the follow-up will continue for 3 more years.

The results of the SELECT have caused much controversy. One hot topic of debate is the formulation of selenium used in the SELECT. Selenomethionine, which was used in the SELECT, can be incorporated non-specifically into proteins in place of methionine [9]. Compartmentation into tissue proteins limits selenomethionine from being further metabolized. On the other hand, monomethylated forms of selenium, including methylseleninic acid (MSA) and methylselenocysteine (MSC), can be easily metabolized to methylselenol, which is considered to be the critical metabolite for the anticancer activity of selenium [9,10]. By itself, methylselenol is highly reactive and difficult to prepare. Therefore, the proximal precursors including MSA and MSC are superior to selenomethionine with regard to providing a steady stream of methylselenol. In fact, studies published before and after the launch of the SELECT have showed that MSA and MSC have stronger anticancer activities than selenomethionine [11–14]. While the conversion of MSC to methylselenol requires the action of β-lyase, MSA can be easily reduced to methylselenol through non-enzymatic reactions involving glutathione (GSH) or NADPH [15]. Due to the fact that epithelial cells express low level of β-lyase, MSA is 10 times more potent than MSC in affecting biological processes *in vitro* [13]. MSA is widely accepted to be the best reagent for delineating the molecular action of selenium in cell culture studies [16–19]. It also has excellent anticancer activity in animals [13,20,21].

In view of the above information, we believe that the potential of selenium compounds as chemopreventive agents for prostate cancer should not be dismissed. Unraveling the mechanisms of action for these agents is urgent and will no doubt be helpful in rational design of future intervention trials. We and others have previously profiled selenium-induced gene expression changes in prostate cancer cells [17,22,23]. Based on the datasets generated from the microarray studies, we conducted a systematic data mining analysis, taking advantage of several publicly available clinical prostate cancer datasets, in order to gain new insights into novel molecular targets that may be relevant to the anticancer activity of MSA [24]. The analysis drew our attention to forkhead box O1 (FOXO1). We found that the expression of FOXO1 is consistently decreased in a large number of prostate cancer specimens, and the microarray analyses showed MSA up-regulates the expression of FOXO1 [24]. FOXO1 is a member of the FOXO family of transcription factors that induces the expression of pro-apoptotic genes including Fas ligand [25,26], bcl-2 family proteins [27–29], and TRAIL [30]. FOXO1 is also involved in cell cycle regulation [31]. FOXO1 is phosphorylated and suppressed by AKT [32,33], which is an important survival molecule for prostate cancer. In prostate cancer cells, androgen receptor (AR) interacts with FOXO1 and inhibits its activation of downstream targets [34]. The current study was designed to examine the role of FOXO1 in mediating the anticancer effect of MSA.

MATERIALS AND METHODS

Materials

MSA was purchased from PharmaSe (Lubbock, TX). Fetal bovine serum, RPMI 1640, and the Lipofectamine PLUS transfection reagents were purchased from Invitrogen (Carlsbad, CA). Immobilon PVDF membrane was purchased from Millipore (Bedford, MA) and ECL Western blotting detection reagent from Amersham Pharmacia Biotech (Arlington Heights, IL). For Western blotting analysis, the antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon (Temecula, CA) and anti-FOXO1 was from Cell Signaling (Danvers, MA). The Cell Death Detection ELISA kit was purchased from Roche Applied Science (Indianapolis, IN). The p3XIRS-luc reporter construct was kindly provided

by Dr. Kun-Liang Guan at the University of Michigan, and the pcDNA3-FKHR expression vector was obtained from Dr. Frederic G. Barr at the University of Pennsylvania. The pcDNA3-AR-FL expression vector was a gift from Dr. Shuyun Yeh at the University of Rochester.

Cell Culture and Treatment

The human LNCaP prostate cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). The LAPC-4 cell line was provided by Dr. Charles L. Sawyers at the University of California at Los Angeles Jonsson Comprehensive Cancer Center. Both LNCaP and LAPC-4 express AR and require androgen for their growth. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM of glutamine. In some experiments, cells were cultured in an androgen-defined condition by using charcoal-stripped FBS in the presence of 1 nM R1881 (a potent synthetic androgen). Treatment with MSA usually began at 72 hr after seeding, when the cultures were 60–80% confluent.

Quantitative Reverse transcription-Polymerase Chain Reaction (qRT-PCR)

The PCR primers and Taqman probes for β-actin, FOXO1, and AR were Assays-on-Demand products from Applied Biosystems (Foster City, CA). The PCR conditions were as follows: an initial incubation at 50°C for 2 min, then a denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The relative quantitation of gene expression was done using the comparative CT ($\Delta\Delta C_T$) method [35]. Details of the procedure were described in our previous publication [22].

Transient Transfection and Reporter Gene Assay

Supercoiled plasmid DNAs were prepared by the Qiagen column procedure (Qiagen, Valencia, CA). Twenty-four hours before transfection, cells were trypsinized and seeded at a density to reach 90–95% confluency at the time of transfection. Transient transfection was carried out by using the Lipofectamine™ and Plus™ reagents (Invitrogen) per instruction of the manufacturer. After incubating with the transfection mixture for 3 hr, the cells were trypsinized and re-plated in triplicate into 6-well plates to achieve equal transfection efficiency. The cells were allowed to attach overnight before 10 μM MSA was added to the culture medium. At 6 or 16 hr following treatment, cells were lysed with 1X Passive Lysis Buffer (Promega, Madison, WI), and the luciferase activity was assayed by using the Luciferase Assay System (Promega). Protein concentration in the cell extract was determined by using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized to the protein concentration of the same sample. The transfection experiments were repeated three times.

Gene Silencing With siRNA

A small interfering RNA (siRNA) designed to target FOXO1 (Cat. # HSS103719) and a matching negative control oligonucleotide were purchased from Invitrogen. These oligonucleotides were transiently transfected into LNCaP cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 hr posttransfection, 10 μM MSA was added to the culture medium and the cells were treated for an additional 24 hr. RNA was prepared from the cells and qRT-PCR was performed to determine the efficiency of gene silencing.

Statistical Analysis

The Student's *t*-test was used to determine significant differences between different groups. Unless otherwise indicated, $P < 0.05$ was considered statistically significant. All analyses were two-tailed.

RESULTS

MSA Induces FOXO1 Expression

We first performed qRT-PCR and Western blotting to confirm the modulation of FOXO1 by MSA in LNCaP cells, as first noted from our microarray analysis [24]. Cells were treated with 10 μM MSA for various lengths of time before they were lysed for RNA and protein purification. The qRT-PCR results are shown in Figure 1a. Induction of FOXO1 mRNA was observed as early as 1 hr after exposure to MSA, suggesting that FOXO1 is a proximal target of MSA. The mRNA level peaked at 2 hr, then declined gradually with time, but still remained elevated at 24 hr. Western blotting of FOXO1 was carried out in LNCaP and LAPC-4 cells (Fig. 1b). No change in protein level was detected until at least after 3 hr. Thus the increases of FOXO1 protein appeared to lag behind the increases of the message, although the protein signal was decidedly stronger by 6 hr in cells treated with MSA.

MSA Induces theTranscriptional Activity of FOXO

As mentioned in the Introduction section, FOXO1 is a transcription factor. In order to study the effect of MSA on the activity of FOXO1 as a transcription factor, we transiently transfected LNCaP and LAPC-4 cells with a luciferase reporter construct, p3XIRS-luc. This

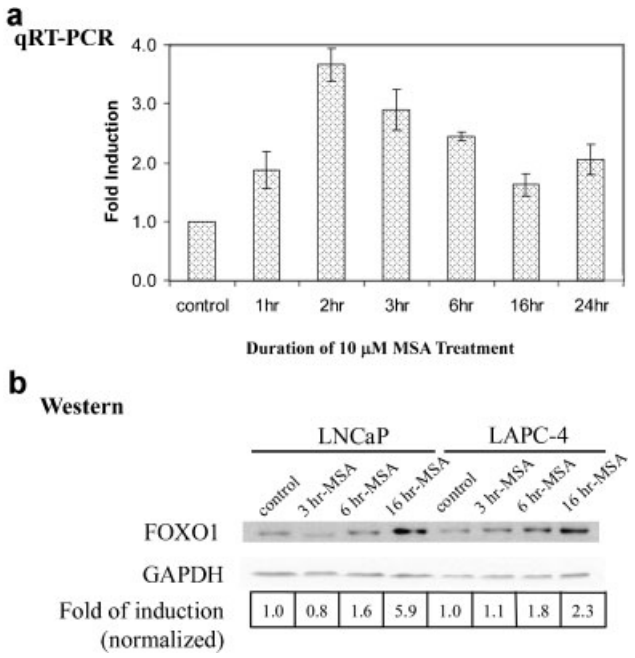

Fig. 1. Effect of methylseleninic acid (MSA) on FOXO1 expression. **a**: Change of FOXO1 mRNA in LNCaP cells as a function of time of MSA treatment, determined by qRT-PCR. The results are shown as mean ± standard error of mean (SEM). **b**: Western analysis of FOXO1 protein level as a function of time of MSA treatment, in both LNCaP and LAPC-4 cells. The band intensity was quantified by volume densitometry and normalized to that of GAPDH. The results were expressed as fold induction over untreated.

construct has three tandem repeats of a FOXO1 binding element, the insulin-responsive sequence (IRS), inserted upstream of the minimal thymidine kinase promoter [33]. It is widely used as an indicator of the transcriptional activity of FOXO proteins. As shown in Figure 2a, the transcriptional activity of this reporter construct was induced by approximately two-fold in LNCaP cells after 6 hr of treatment with 10 μM MSA. A pronounced induction (>5-fold) was observed in LNCaP after 16 hr of treatment ($P < 0.01$). Nearly identical results were obtained in LAPC-4 cells (Fig. 2b).

FOXO1 Gene Silencing Blocks MSA-Induced Apoptosis

MSA has been shown to induce apoptosis in prostate cancer cells by several groups, including ours [17–19,36]. The experiments described above suggested that MSA induces the FOXO1 signaling pathway, which is known to positively regulate apoptosis. To establish the role of FOXO1 in MSA-induced apoptosis, we employed the RNA interference technique to knockdown the expression of FOXO1. A commercially available siRNA targeting FOXO1 was obtained. To confirm the specificity of the siRNA, we performed a Local Alignment Search Tool (BLAST) against the entire human transcriptome using the sequence provided by the manufacturer. With the exception of FOXO1, the search identified no other homology with the siRNA sequence, including other FOXO members. When introduced into LNCaP cells, the FOXO1 siRNA, named siFOXO1 hereafter, was able to decrease the baseline expression of FOXO1 by approximately 50% (Fig. 3a). Consistent with our previous finding, a two-fold induction of FOXO1 was observed when the cells were treated with 10 μM MSA for 24 hr (comparing columns 1 and 3). siFOXO1 was able to abolish this induction by MSA (comparing columns 3 and 4).

Apoptosis was quantitated in siRNA-transfected and MSA-treated cells by using an ELISA-based

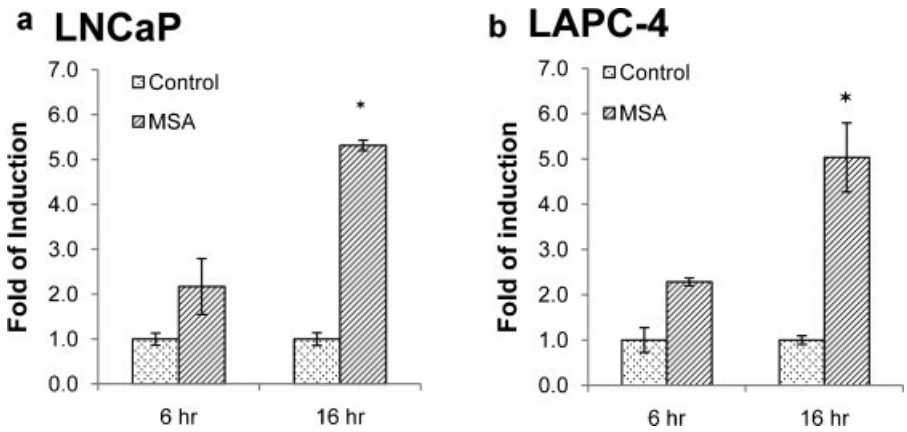

Fig. 2. Induction of FOXO transcriptional activity by MSA. LNCaP (**a**) and LAPC-4 (**b**) cells were transfected with the p3XIRS-luc construct and treated with 10 μM MSA for the indicated times. At the end of treatment, cells were lysed for luciferase assay. Total protein concentration was also determined and used to normalize the luciferase reading. The results were expressed as mean ± SEM; *, $P < 0.05$.

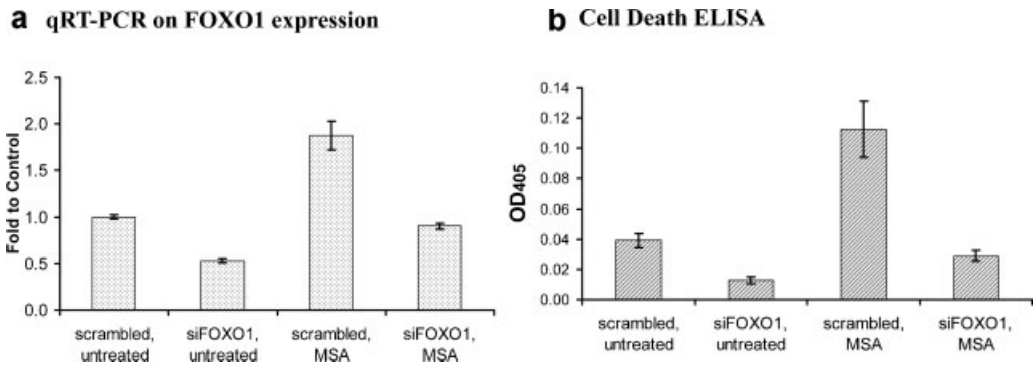

Fig. 3. Effect of FOXOI gene silencing on MSA-induced apoptosis. **a**: qRT-PCR analysis of FOXOI expression in cells transfected with small interference RNAs (siRNAs) and treated with or without MSA. The data were expressed as fold relative to the scrambled, untreated control. **b**: Quantitation of apoptotic cell death by an ELISA method.

method. The result is shown in Figure 3b. In general, the level of apoptosis in these cells correlated well with the expression level of FOXO1 (Fig. 3a), confirming that FOXO1 plays an important role in apoptosis regulation. More importantly, when the induction of FOXO1 was blocked by the addition of siFOXO1, no induction of apoptosis was observed (Fig. 3b, comparing columns 1 and 4). These results suggest that FOXO1 is a key mediator of apoptosis induction by MSA.

FOXO1 Activation Suppresses AR *trans*-Activation

To examine the effect of FOXO1 activation on the transcriptional activity of AR, we transiently co-transfected LNCaP cells with a reporter construct containing three repeats of the androgen response element (ARE) ligated in tandem to the luciferase reporter, together with a FOXO1 expression vector, pcDNA3-FKHR, or the empty vector. The ARE-luciferase reporter assay is commonly used to assess the *trans*-activating activity of AR. Following transfection, cells were exposed to 1 nM R1881, a synthetic androgen, for 6 or 16 hr before they were lysed for luciferase assay. As shown in Figure 4a, the AR transcriptional activity was greatly stimulated by the addition of the ligand. In the presence of ectopically expressed FOXO1, the induction was significantly diminished (Fig. 4a, comparing columns 2 and 4 for both time points). Therefore, our results confirmed published studies showing that FOXO1 activation suppresses AR signaling [37–40].

FOXO1 Induction Contributes to AR Suppression by MSA

It has been found previously that MSA is a potent suppressor of AR signaling [22,23,41]. The mechanisms involved in suppression of AR signaling by MSA include reduction in AR mRNA transcription [22,41] and stability, increase in AR protein turnover,

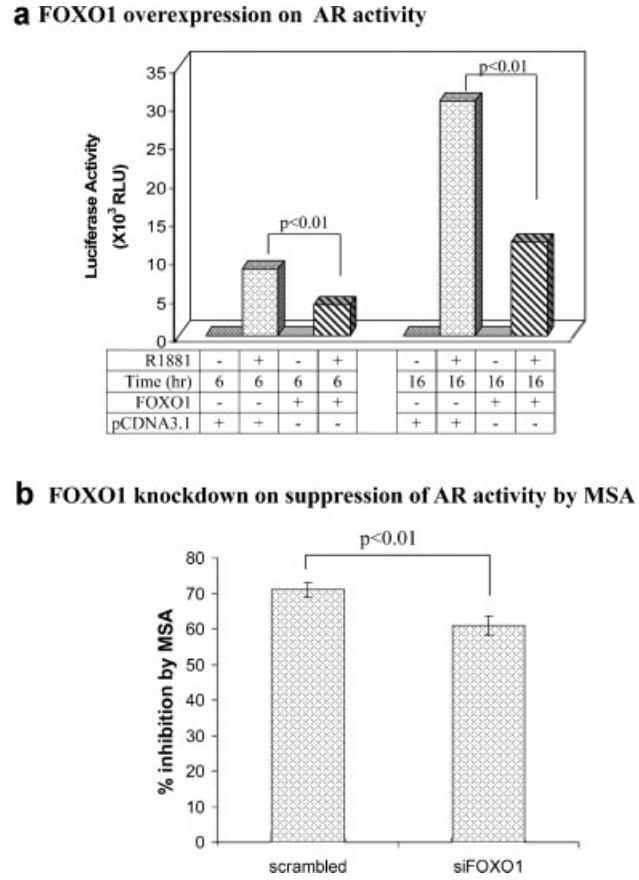

Fig. 4. Induction of FOXOI contributes to AR suppression by MSA. **a**: Increased expression of FOXOI reduced the transcriptional activity of AR. LNCaP cells were co-transfected with the ARE-luciferase reporter construct and either the pcDNA3-FKHR or the pcDNA3 vector, and treated with 1 nM RI88I for the indicated times. **b**: FOXOI knockdown attenuated the suppression of AR trans-activation by MSA. LNCaP cells were co-transfected with the ARE-luciferase construct and either the scrambled control or siFOXOI, and treated with 10 μM MSA for 24 hr. The luciferase reading was normalized by protein concentration. The experiment was done three times and the results were expressed as mean percent inhibition ± SEM.

reduction in AR translocation, inhibition of co-activator recruitment, and increased co-repressor recruitment to the promoters of AR-regulated genes [22,41,42]. The result from the previous section prompted us to investigate whether FOXO1 induction is a contributing factor for AR suppression by MSA. Once again, we employed the gene knockdown approach. LNCaP cells were co-transfected with the ARE-luciferase construct and siFOXO1, and treated with 0 or 10 μM MSA. In the presence of the scrambled oligo, MSA suppressed AR activity by approximately 70% (Fig. 4b). This is in line with our previous observations [22]. However, when FOXO1 was silenced, the suppression was attenuated to about 60% ($P < 0.01$). This was further confirmed when we examined the modulation of prostate-specific antigen (PSA) expression by MSA in the presence or absence of siFOXO1 (data not shown). These results are in agreement with previous studies showing that MSA suppresses AR signaling through a multitude of mechanisms and identified FOXO1 activation as a novel mechanism contributing to the inhibition of AR *trans*-activation by MSA.

DISCUSSION

Despite the protective effect of selenium against prostate cancer demonstrated by the NPC study and several studies which showed selenium is very effective in switching off androgen signaling, recent results from the SELECT showed that selenium, alone or in combination with vitamin E, did not prevent prostate cancer in a randomized trial of 33,000 men at average risk [8]. Several potential reasons have been discussed to explain the discrepancy of the findings in SELECT and the NPC trial. In addition to the dose and formulation of selenium used in the trial, one important consideration is the baseline selenium level. The NPC trial showed that the protective effect of selenium was limited to patients with baseline serum selenium in the lower two tertiles [7]. The average baseline selenium level of the participants in SELECT was much higher than that observed in the NPC study. In fact, 78% of men in SELECT had baseline selenium above the range that selenium provided protection in the NPC trial (<121.6 ng/ml) [8]. Another important consideration is how selenium exerts its anticancer activity. The Physicians' Health Study demonstrated an inverse association of plasma selenium level with risk of advanced prostate cancer, not localized prostate cancer, suggesting selenium might function by slowing down tumor progression [5]. In view of the above information, we believe that the negative finding by SELECT should not be simply interpreted as selenium is ineffective against prostate cancer. Instead, the outcome of this trial, as well as those of several recently published clinical trials [43–45], may indicate that it is difficult to find a single chemoprevention strategy which can benefit the general population. There is an urgent need to re-evaluate all the pre-clinical and clinical evidence to identify the subset of patients that are most likely to benefit from selenium supplementation.

This report is the first to show that MSA induces the expression of FOXO1. The elevated expression is accompanied by an increase of the FOXO transcriptional activity. We further demonstrated that FOXO1 is a key mediator of apoptosis induction by MSA. The above conclusion is supported by the following observations. First, FOXO1 induction occurred very early following MSA treatment, suggesting that FOXO1 is a proximal target of MSA. Second, MSA failed to induce apoptosis when FOXO1 stimulation was abolished by the addition of a FOXO1-specific siRNA. 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). 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. As a pro-apoptotic member of the Bcl-2 family, Bim functions by antagonizing the actions of the anti-apoptotic Bcl-2 and Bcl-XL. Both TRAIL and TRADD are associated with the extrinsic pathway. MSA has been shown to activate caspases that are involved in both the intrinsic and extrinsic apoptosis signaling pathways [18,36]. We are currently working on identifying the pro-apoptotic targets of FOXO1 that are induced by MSA. In addition to its role in regulating apoptosis, FOXO1 also plays an important role in cell cycle control. It up-regulates the expression of p27 [31,46] and down-regulates the expression of cyclins D1 and D2 [47,48], a pattern consistent with the G1 cell cycle block by MSA [17,49]. Therefore, it is possible that FOXO1 also mediates the cell cycle effects of MSA. Research along this line is currently ongoing in our laboratories.

Several mechanisms could account for the induction of FOXO1 signaling by MSA. One is through the induction of FOXO1 expression, as evidenced by the increased transcript and protein levels following MSA treatment. It has been shown that AR interacts and suppresses the activity of FOXO1 in prostate cancer cells [34,50]. Another potential mechanism of MSA activation of FOXO1 is through decreasing AR expression and thereby relieving the inhibition of FOXO1 by AR. This is supported by the fact that ectopic expression of AR could attenuate the induction of FOXO1 activity by MSA (data not shown). Yet, there might be a third mechanism by which MSA induces FOXO1. A key regulator of cellular FOXO1 activity is

Akt, an important survival molecule for many cancer types, including prostate cancer. Akt phosphorylates FOXO1, which leads to nuclear exclusion and proteosomal degradation of FOXO1 [33]. MSA has been shown to suppress the PI3K-Akt signaling pathway [19,51–53]. Therefore, it is possible that relieving the suppression by Akt may contribute to MSA induction of FOXO1. Further experimental evidence is needed to support this hypothesis.

In agreement with previous reports [37,38,40,54], our data showed increased abundance of FOXO1 leads to decreased AR activity. Together with the well-documented AR inhibition of FOXO1 activity, it appears that in prostate cancer cells, the AR and FOXO1 signaling pathways antagonize the action of each other. The outcome is likely determined by the relative abundance of AR and FOXO1 proteins. When AR signaling dominates, the growth inhibitory signals conveyed by FOXO1 are muted, and the cells undergo proliferation. On the other hand, when FOXO1 signaling dominates, the antiproliferative and pro-apoptotic signaling prevail. When prostate cancer cells are exposed to MSA, AR signaling is suppressed whilst FOXO1 signaling is stimulated. By doing so, MSA could shift the balance heavily in favor of FOXO1, leading to cell cycle arrest and apoptosis. Therefore, modulating the crosstalk between AR and FOXO1 could be the key mechanism underlining the anticancer effect of MSA in the prostate.

CONCLUSIONS

The work described herein demonstrates that MSA activates FOXO1 signaling pathway. FOXO1 plays a critical role in mediating the apoptotic activity of MSA, and also contributes to the suppression of androgen signaling by MSA. This study enhances our understanding of the molecular mechanisms of the anticancer activity of MSA, which will be critical for designing future prostate cancer intervention studies with MSA.

ACKNOWLEDGMENTS

This work was supported by DOD New Investigator grant no. W81XWH-05-1-0598 (H. Zhang), American Cancer Society grant no. RSG-07-218-01-TBE (Y. Dong), National Cancer Institute grant nos. K01 CA114252 (Y. Dong), P01 CA126804 (C. Ip, H. Zhang, and Y. Dong), start-up funds from the Louisiana Cancer Research Consortium (H. Zhang and Y. Dong).

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