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Manuscript accepted by Cancer

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 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 FOXO1A in mediating the anticancer effect of selenium. For Aim 1, we originally proposed to use dutasteride, a proprietary drug of GlaxoSmithKlein (GSK), as the 5α -reductase inhibitor. We submitted a research proposal to GSK to request the compound (not funding) after the grant was recommended for funding. However, after much delay, no agreement was reached between Roswell Park and GSK because the company insisted on full ownership of all intellectual properties and research products, even though the study was designed solely by investigators at Roswell Park with no input from GSK. Our Technology Transfer Office felt that we could not possibly accede to this demand. Finasteride, despite being an older drug, has several advantages over dutasteride. First, its efficacy has been proven by a phase III trial, whereas dutasteride is being tested and the result will not be available for a few years. Second, there are minimal side effects with long-term finasteride treatment. No such information is available with dutasteride. Third, finasteride is available commercially from Steraloids (Newport, RI). Based on these considerations, we have decided to use finasteride to conduct a proof-of-principle study. We were able to generate very encouraging data with the finasteride and selenium combination (see the following section). The experiments can be easily duplicated with dutasteride once encouraging clinical data are available.

B. BODY

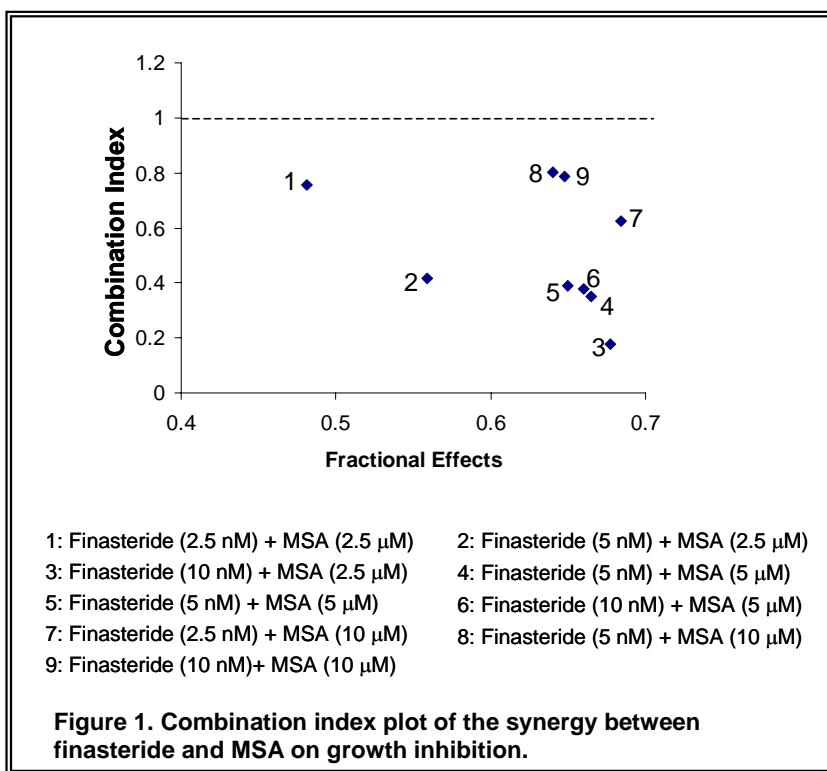
Task 1. Evaluate the efficacy of selenium and finasteride combination on cell growth in cell culture

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 μ M 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 Calcsyn software (Biosoft). This program uses the median-effect 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.

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

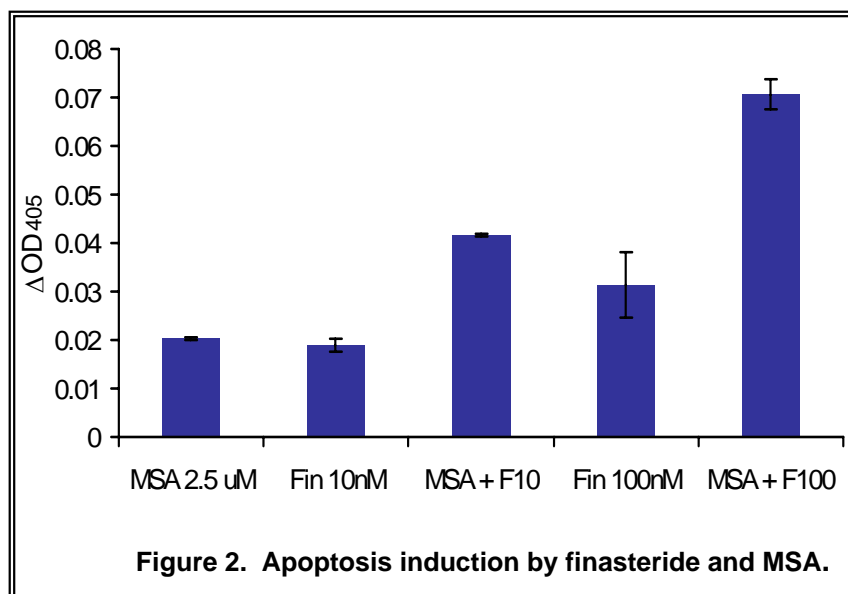
A combination index (CI) of < 1 , 1 or > 1 denotes synergism, additivity or antagonism, respectively. Each spot in Fig. 1 corresponds to the combination number shown beneath the plot. All nine combinations produced a CI value of less than 1, suggesting a synergy between finasteride and MSA in cell growth inhibition.



The smaller the CI value, the better is the synergistic effect. Combination 3 (10 nM finasteride and 2.5 μ M 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.

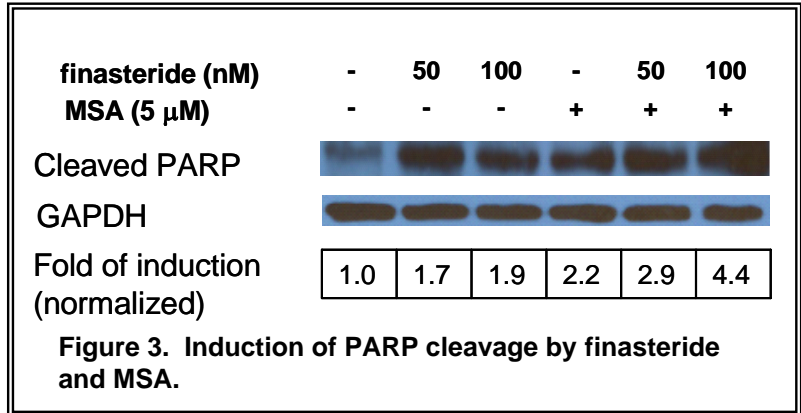
Synergy of finasteride and MSA in apoptosis induction

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 μ M 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 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).

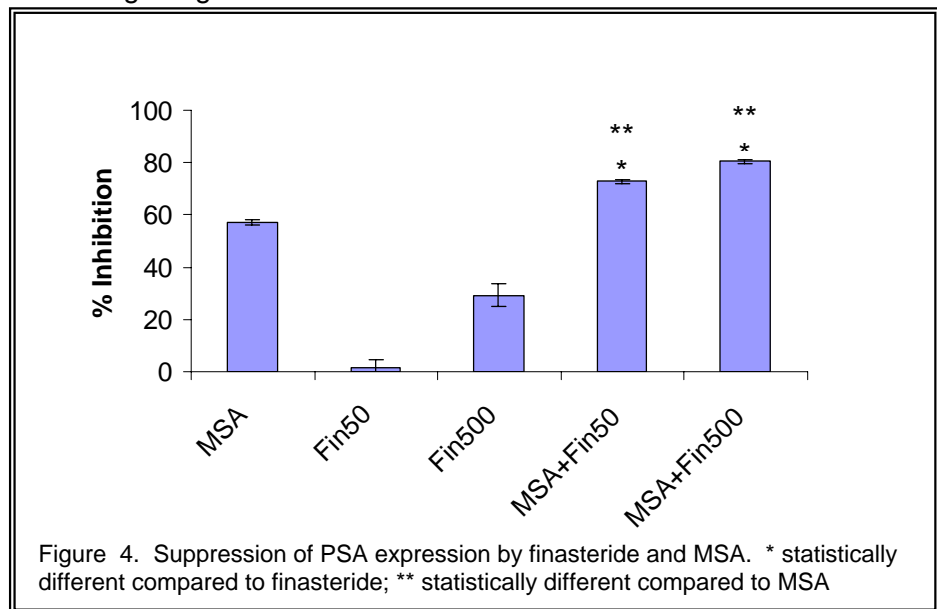
To study the effect of finasteride and MSA on PARP cleavage, a characteristic indicative of caspase activation, LNCaP cells were treated with 0, 50, or 100 nM finasteride for 32 hr, and 0 or 5 μ M MSA for an additional 16 hr. Whole cell lysate was prepared and Western blotting was performed using an 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 μ M 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.

Augmented suppression of androgen signaling by finasteride and MSA

To study the effect of finasteride and MSA on the expression of PSA, LNCaP cells were cultured in phenol-red free RPMI 1640 medium containing 10% charcoal-stripped serum and 10 nM testosterone. The reason for using a higher concentration of testosterone than the 1 nM used in the previous section is for optimal induction of PSA. The cells were treated with 50 or 500 nM finasteride for 24 hr, 2.5 μ M MSA for 6 hr, or the combination. The expression of PSA was determined by quantitative RT-PCR (qRT-PCR). The results, expressed as percentage inhibition relative to untreated control, are shown in Fig. 4. MSA alone suppressed PSA by 57%. Finasteride at



50 nM did not affect PSA expression, but combined with MSA resulted in 73% inhibition. Increasing the finasteride concentration to 500 nM led to a 30% inhibition, and the combination with MSA depressed PSA by more than 80%. In both cases, the differences between the combination effect and the single agent effects were statistically significant ($p < 0.001$). This result demonstrates an augmented suppression of androgen signaling when the two agents are used in combination.

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

In order to study the effect of MSA on the transcriptional activity of FOXO1A, we transiently transfected LNCaP and LAPC-4 cells with a luciferase reporter construct, p3xIRS-luc. This construct has 3 tandem repeats of a FOXO1A binding element, the insulin-responsive sequence (IRS), inserted upstream of the minimal thymidine kinase promoter (3). It is widely used as an indicator of the transcriptional activity of FOXO1A. Following transfection, cells were trypsinized, re-plated and were allowed to attach overnight before the addition of 10 μ M MSA to the culture medium. At 6 or 16 hr, the cells were lysed 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 5, the transcriptional activity of FOXO1A was induced by 2-fold after 6 hr of MSA treatment. A greater than 5-fold induction was observed after 16 hr.

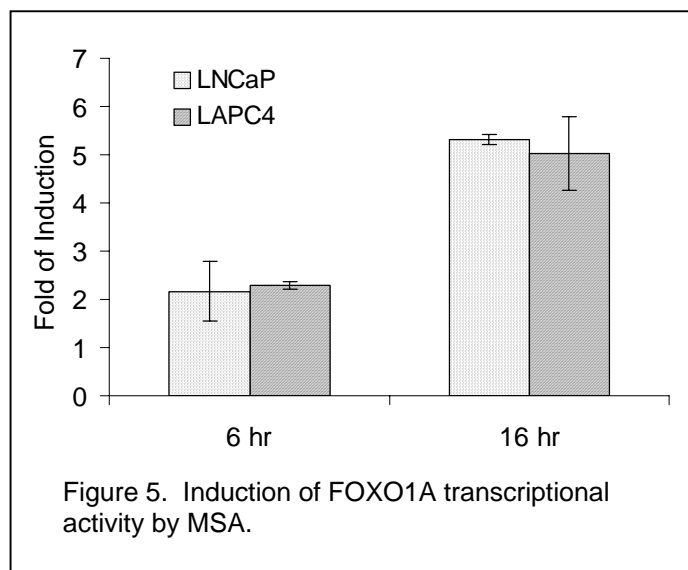


Figure 5. Induction of FOXO1A transcriptional activity by MSA.

In the proposal, we alluded to the possibility that finasteride may potentiate induction of FOXO1A activity by MSA since the interaction between AR and FOXO1A is androgen-dependent. Therefore, in the presence of finasteride and MSA, the DHT-AR complex should be 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 FOXO1A transcriptional activity, we transfected LNCaP cells with the p3xIRS construct as described above.

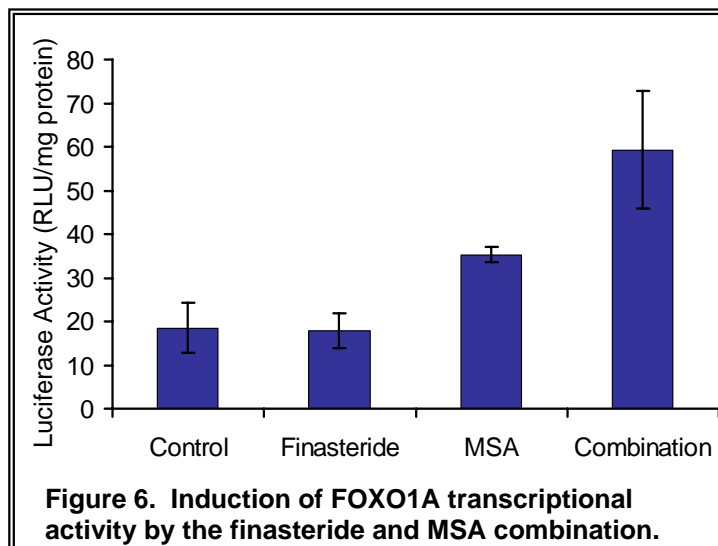


Figure 6. Induction of FOXO1A transcriptional activity by the finasteride and MSA combination.

Cells were treated with 10 nM finasteride, or 5 μ M MSA, or both. As shown in Fig. 6, finasteride alone had no effect on FOXO1A 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 exaggerated increase of FOXO1A transcriptional activity, suggesting a cooperative interaction between the two drugs.

Task 5. Evaluate the effect of FOXO1A knockdown on the growth inhibitory action of selenium

To further establish the role of FOXO1A in MSA-induced apoptosis, we employed the RNA interference (RNAi) technique to knockdown the expression of FOXO1A. A small interfering RNA (siRNA) designed against FOXO1A (siFOXO1A) 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 μ M 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. 7, siFOXO1A was able to decrease the baseline expression of FOXO1A by approximately 50%. Consistent with our previous finding, a 2-fold induction of FOXO1A was observed when the cells were treated with MSA for 24 hr (comparing columns 1 and 3). This induction was abolished when siFOXO1A was present (comparing columns 3 and 4).

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.

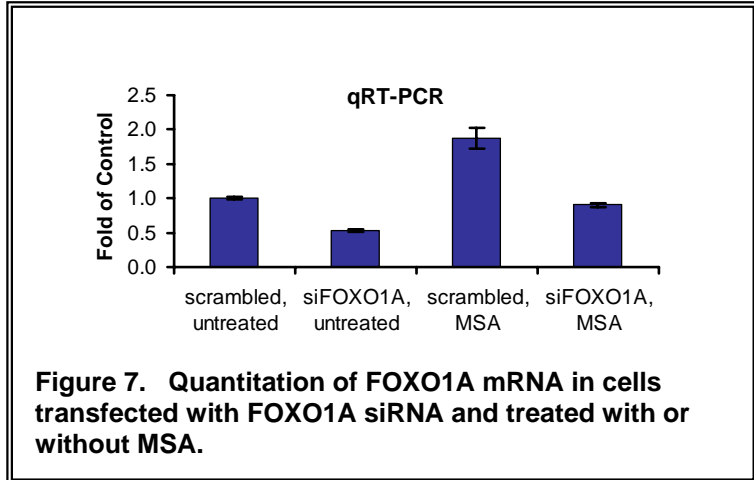


Figure 7. Quantitation of FOXO1A mRNA in cells transfected with FOXO1A siRNA and treated with or without MSA.

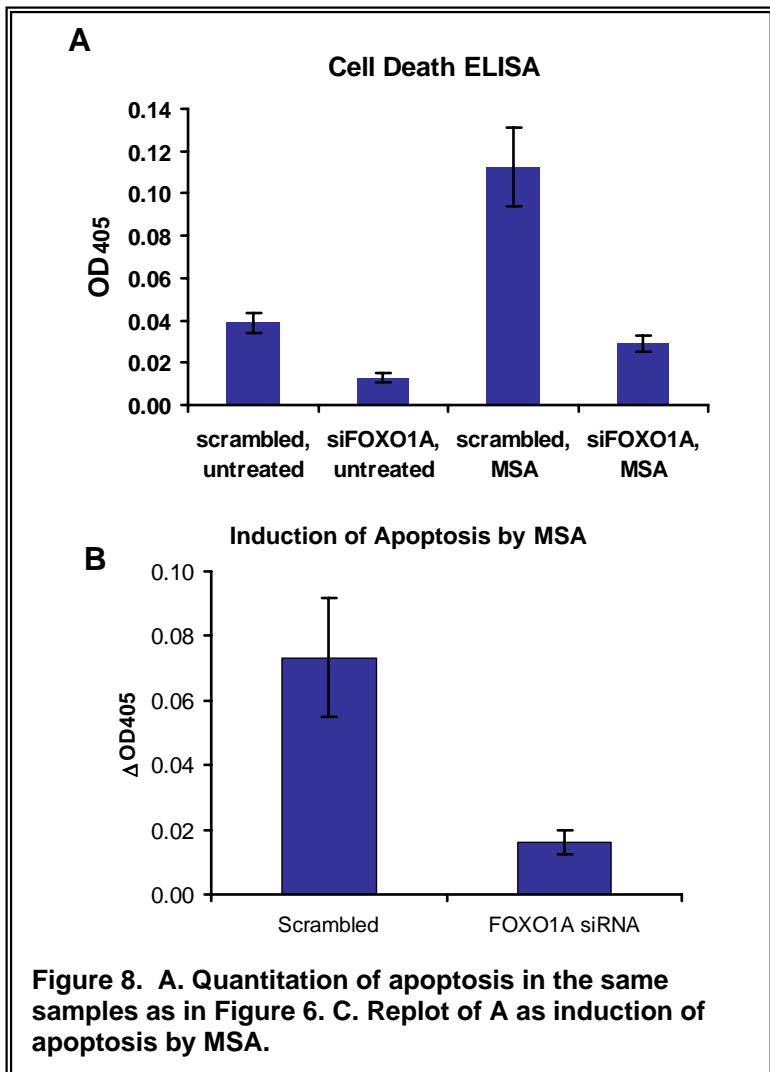


Figure 8. A. Quantitation of apoptosis in the same samples as in Figure 6. C. Replot of A as induction of apoptosis by MSA.

As shown in Fig. 8A, FOXO1A knockdown decreased both the baseline and MSA-induced apoptosis. The level of apoptosis correlated well with the expression level of FOXO1A (Fig. 7), suggesting that FOXO1A is critical for both MSA-dependent and –independent apoptosis. More importantly, in the absence of FOXO1A siRNA, MSA increased apoptosis by 0.075 OD₄₀₅ units (Fig. 8B). However, in the presence of FOXO1A siRNA, the increase was reduced to 0.016 units (Fig. 8B). Similar results were also obtained in LAPC-4 cells (data not shown).

Task 6. Determine whether AR overexpression could mitigate the modulation of FOXO1A activity by selenium

To determine whether selenium induction of FOXO1A 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 FOXO1A 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.

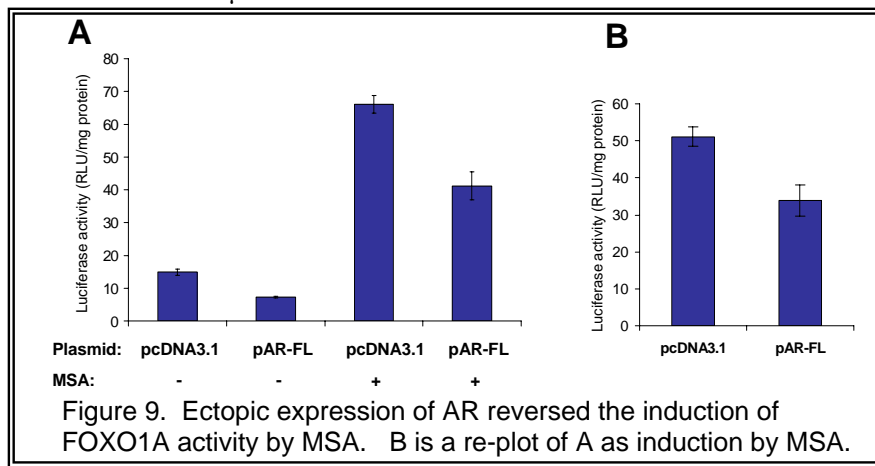


Figure 9. Ectopic expression of AR reversed the induction of FOXO1A activity by MSA. B is a re-plot of A as induction by MSA.

Total protein concentration was also determined to normalize the luciferase result. As shown in Figure 9A, ectopic expression of AR diminished both the baseline and MSA-induced FOXO1A trans-activation activity. This is consistent with previously published reports that AR negatively regulated FOXO1A activity (4,5).

When re-plotted as induction by MSA (Figure 9B), it is obvious that MSA induction of FOXO1A activity was partially reversed in cells expressing exogenous AR. This result suggests that AR indeed plays a role in the induction of FOXO1A activity by MSA.

C. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that the combination of MSA and a 5α-reductase inhibitor (finasteride) has a synergistic effect in inhibiting the growth of prostate cancer cells.
- Demonstrated that growth inhibition by the MSA and finasteride combination could be attributed, at least in part, to their synergy in apoptosis induction.
- Proved that the MSA and finasteride combination leads to a greater suppression of androgen signaling than either agent alone.
- Using a luciferase reporter construct, demonstrated MSA induces the transcriptional activity of FOXO1A, and confirmed the possibility that finasteride potentiates FOXO1A induction by MSA.

- Through FOXO1A knockdown, demonstrated that FOXO1A plays an important role in mediating apoptosis induction by MSA.
- Confirmed that MSA induction of FOXO1A activity is mediated in part by decreasing AR expression and therefore eliminating the inhibitory effect of AR on FOXO1A.

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*, in press.

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".

Funding applied

1. American Cancer Society Research Scholar Grant, "Enhancing the Chemopreventive Efficacy of Finasteride by Selenium (H. Zhang, PI)", submitted April 2006.
2. National Institute of Health P01, "Translational research of finasteride and selenium prevention of prostate cancer (H. Zhang, Co-Project Leader)", submitted June 2006.

E. CONCLUSIONS

The results from the current study demonstrated a synergism between a 5 α -reductase inhibitor, finasteride, and MSA in arresting the growth of prostate cancer cells. The growth inhibition could be attributed, at least in part, to apoptosis induction by these agents. 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.

The current study also established a role of FOXO1A in mediating apoptosis induction by selenium. In addition to inducing FOXO1A transcription directly, we now have evidence to

support an indirect mechanism by which MSA activates FOXO1A. By depression AR expression, MSA could relieve the inhibitory effect of AR on FOXO1A, therefore further increases FOXO1A activity.

As described in the Introduction, RPCI could not reach an agreement with GSK that is acceptable to both parties, and therefore we could not obtain the proprietary drug dutasteride to conduct the proposed research. However, we were able to generate very promising results using a different 5 α -reductase inhibitor, finasteride, which is available commercially. We propose to conduct the rest of the *in vitro* and *in vivo* experiments using finasteride to replace dutasteride.

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**Augmented Suppression of Androgen Receptor Signaling by a
Combination of α -Tocopheryl Succinate and Methylseleninic Acid**

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CONDENSED ABSTRACT

Treatment of LNCaP human prostate cancer cells with a combination of α -tocopheryl succinate (α TS) and methylseleninic acid (MSA) depressed androgen receptor (AR) and prostate specific antigen (PSA) mRNA levels by 60% and 90%, respectively. Each agent by itself was much less effective. The results suggest an opportunity to exploit the cooperativity between α TS and MSA in blocking androgen signal transduction as a means to prostate cancer control.

ABSTRACT

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

METHODS. LNCaP cells were treated with α TS alone for 31 hr, MSA alone for 3 hr, or α TS first for 28 hr and α TS/MSA together for the last 3 hr. AR and PSA mRNA levels were quantitated by qRT-PCR. AR trans-activation was determined by the ARE-luciferase reporter assay. Both cellular and secretory PSA was also measured by an ELISA method.

RESULTS. Different doses of α TS were evaluated in combination with MSA. Some striking results are highlighted below for α TS alone, MSA alone, or α TS/MSA (presented in this order). AR mRNA level was depressed by 0%, 20%, or 60%, respectively; AR trans-activation was inhibited by 35%, 10% or 60%, respectively; while 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 α TS/MSA produced a greater than additive effect in suppressing AR signaling compared to the single agent. Decreased AR abundance is a major factor, but not necessarily the sole factor, in diminishing the transcriptional activity of AR by α TS or MSA.

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

INTRODUCTION

There is extensive documentation that androgen is required for the development of prostate cancer in humans.¹ Testosterone and dihydrotestosterone (DHT) are the two key androgens in men. Since DHT binds to the androgen receptor (AR) with a greater affinity than does testosterone, it is the more potent androgen in a biological sense. Steroid 5 α -reductase is the enzyme responsible for catalyzing the irreversible conversion of testosterone to DHT.² Many synthetic inhibitors of 5 α -reductase have been developed, although only one, viz. 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 non-toxic agents capable of disrupting androgen signaling beyond the 5 α -reductase step would be highly desirable.

Recently, α -tocopheryl succinate (α TS) 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 two treatments. The effect of MSA is acute, whereas the effect of α TS is delayed. These observations imply that MSA and α TS may have different mechanisms in down-regulating AR level. In the present study, we carried out a series of experiments to investigate whether α TS and MSA in combination would produce an augmented effect. We analyzed AR message and protein levels as well as AR trans-activating 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 following α TS/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 μ g/ml of streptomycin, and 2 mM of glutamine. Treatment with α TS or MSA began at 72 hr or 100 hr after seeding, respectively, when the cultures reach a confluency of 60-80%. Cells were harvested after 31 hr if treated with α TS alone, or after 3 hr if treated with MSA alone. If cells were treated with the combination of α TS and MSA, they were exposed first to α TS for 28 hr, and then to MSA for the last 3 hr (with α TS still present in the medium) before harvesting. These time points were chosen based on prior studies of the duration required to achieve a suppression of AR signaling by MSA or α TS as a single agent.⁴⁻⁷ A significant downregulation of AR protein level is readily detectable between 3 and 6 hr of MSA treatment. In contrast, an exposure time of 24 to 48 hr to α TS is normally necessary to suppress AR signaling. The experiment was repeated three times, and the RNA and cell lysates collected and subjected to real-time RT-PCR or Western analysis, respectively. α TS 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 β -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 three 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 semi-quantitative 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 two 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 hr, the cells were trypsinized, resuspended in a medium containing charcoal-stripped serum and 10 nM DHT (Sigma, St. Louis, MO), and plated in triplicate onto 6-well plates. Cells were allowed to recover for 24 hr 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 three 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 three separate experiments \pm standard error.

PSA Measurement by ELISA

The cell cultures were prepared slightly differently for PSA measurement by ELISA as reported previously.⁵ At 72 hr after seeding, cells were rinsed three times with 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 h of incubation. The washing brought the level down to within 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 three 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 Inc. (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's two-tailed *t* test was used to determine significant differences between treatment and control values, and $P < 0.05$ was considered statistically significant.

RESULTS

Dose Response of AR Down-Regulation by α TS or MSA

In order to select the appropriate dose of α TS and MSA to use in the combination, it was important to first find out the sensitivity of AR to each agent. We tested α TS at 20, 30 or 40 μ M, and MSA at 2.5, 5 or 10 μ M, respectively. As noted in the Methods section, cells were harvested at 31 hr after α TS treatment, and at 3 hr after MSA treatment. AR level was quantified by real-time RT-PCR. α TS reduced AR expression by 0%, ~10% or ~60% at concentrations of 20, 30 or 40 μ M, respectively (data not shown). We repeated this experiment a number of times, and confirmed that the AR dose response to α TS was apparently very steep between 30 to 40 μ M. On the other hand, MSA reduced AR expression by ~20%, ~40% or ~60% at concentrations of 2.5, 5 or 10 μ M, respectively (data not shown). Thus the AR dose response to MSA was linear in this range. The above information was helpful in deciding on 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 μ M α TS/2.5 μ M MSA or 40 μ M α TS/2.5 μ M MSA for the combination experiments.

Combined Effect of α TS/MSA Treatment on AR Depression

Cells were treated with α TS first for 28 hr, followed by α TS and MSA for 3 more hr before harvesting. We did not change the medium at the time MSA was added. The single agent culture was treated with either α TS alone for 31 hr, or MSA alone for the last 3 hr. The quantitative RT-PCR AR level from the 31-hr untreated control culture was set at 100%. The AR data from the three treated cultures (α TS alone, MSA alone, α TS + MSA) are expressed as % of control as shown in Fig. 1A. α TS at 20 μ M had no effect on AR mRNA level, while MSA at 2.5 μ M

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 AR level to 10% of control. The Western blot data of the second experiment are shown in Fig. 1B. The protein results are also expressed as % 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 since 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 Trans-Activating Activity Inhibition

Low abundance of AR is expected to diminish AR trans-activation. The ARE-luciferase reporter assay is commonly used to assess AR trans-activating activity. Fig. 2 shows the results of the effects of αTS /MSA with this assay. The data are also expressed as % of untreated control. αTS alone at 40 μM decreased AR activity to ~65% of control, while MSA alone at 2.5 μM produced only a 10% inhibition at best. The combination, on the other hand, depressed AR activity to ~40% of control. The results are congruent with the interpretation that the inhibition of AR trans-activation 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 trans-activation 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 , since 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.

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 two combinations: 20 μM αTS /2.5 μM MSA, or 40 μM αTS /2.5 μM MSA. The results, which are expressed as % of untreated control, are shown in Fig. 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 expression 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 hr of treatment.^{4,11} In assessing the response of AR to αTS and MSA, the cells were treated with αTS first for 28 hr, followed by αTS and MSA for another 3 hr. 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 two agents may work cooperatively in modulating AR expression.

Our previous report showed that over-expression of AR weakened considerably 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 downregulation 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 on 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 α TS- or MSA-treated cells reveals additional information of interest. α TS at 20 μ M 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 μ M 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 α TS or MSA. AR signaling 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 co-activators or co-repressors.¹³ α TS and MSA may potentially modulate one or more of these steps (unpublished observations by Y. Dong).

The ongoing SELECT trial is testing the efficacy of selenium and/or α -tocopheryl acetate in prostate cancer prevention.¹⁴ α TS 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 α TS, rendering α TS more hydrophilic than α -tocopherol. It is generally believed that α TS is taken up more efficiently by cells than α -tocopherol. More than a decade ago, Turley *et al.*¹⁵ showed that α TS at a concentration of 30 μ M 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 μ M and 200 μ M, 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 a 72 hr 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 α TS. Therefore, it remains to be determined whether α -tocopherol or α -tocopherol acetate may induce growth inhibition and suppress AR signaling following a prolonged duration of 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 supra-physiological 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 non-enzymatic reaction. The cellular and molecular responses of prostate cells to physiological 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.

FIGURE LEGENDS

Figure 1. Effect of α TS and/or MSA on AR expression. A. Changes in AR mRNA level as determined by real time RT-PCR. B. Western blot data of changes in AR protein level. The values represent mean \pm SE (n=3). *Statistically different from the untreated control, P<0.05. **Statistically different from either of the single agent treatment, P<0.05.

Figure 2. Effect of α TS and/or MSA on ARE-luciferase activity. The values represent mean \pm SE (n=3). *Statistically different from the untreated control, P<0.05. **Statistically different from either of the single agent treatment, P<0.05.

Figure 3. Effect of α TS and/or MSA on PSA expression. A. Changes in PSA mRNA level as determined by real time RT-PCR. B. Western blot data of changes in cellular PSA protein level. The values represent mean \pm SE (n=3). *Statistically different from the untreated control, P<0.05. **Statistically different from either of the single agent treatment, P<0.05.

Figure 4. Effect of α TS and/or MSA on cellular and secretory PSA as determined by ELISA. The values represent mean \pm SE (n=3). *Statistically different from the untreated control, P<0.05. **Statistically different from either of the single agent treatment, P<0.05. ***Statistically different from α TS treatment only, P<0.05.

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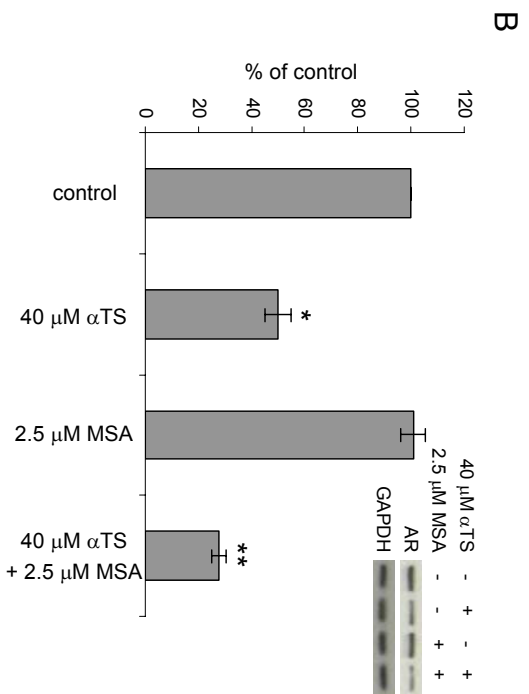
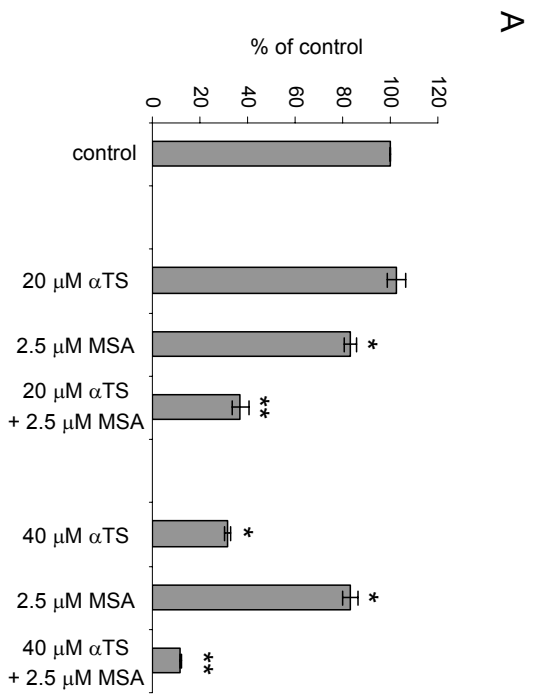


Figure 1

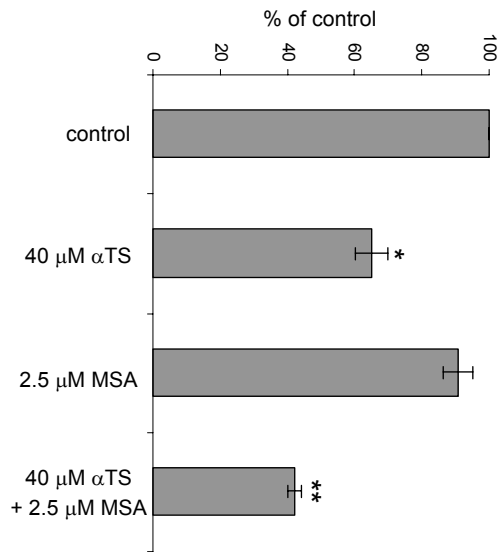


Figure 2

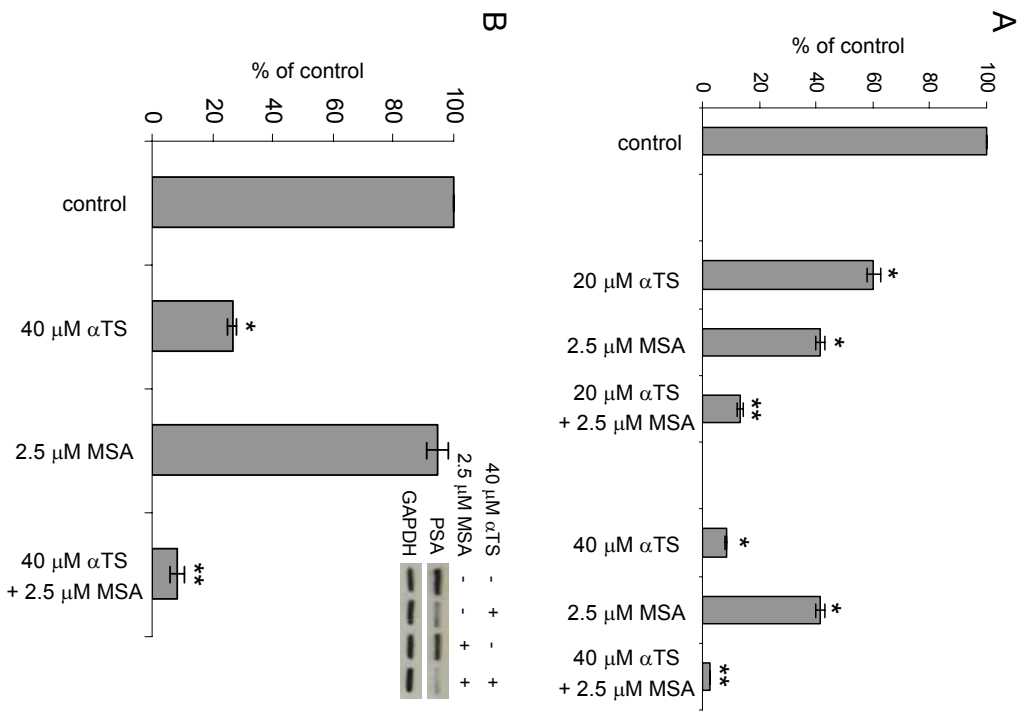


Figure 3

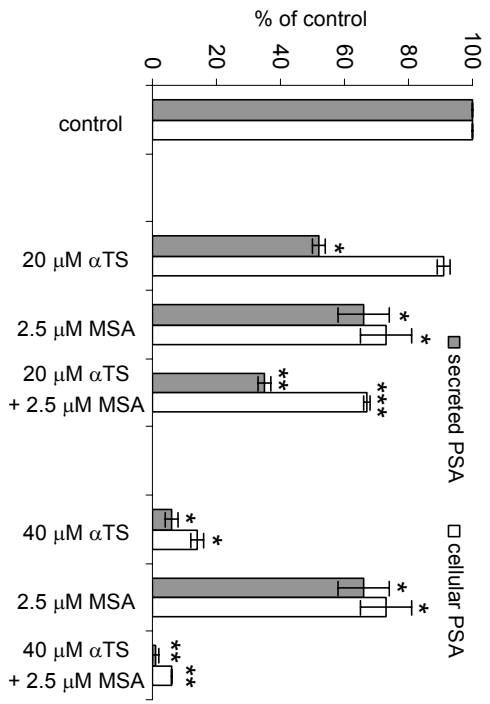


Figure 4