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Chemoprevention of Prostate Cancer

PRINCIPAL INVESTIGATOR: Shuang Liu, Ph.D.

CONTRACTING ORGANIZATION: Tulane University
New Orleans, LA 70112

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14. ABSTRACT The project is to investigate telomerase as a potential target of AR signaling suppression by selenium. We found that over-expression of hTERT attenuates the apoptosis inducing activities of selenium, supporting an important role of hTERT in selenium action in prostate cancer cells. More importantly, we found that combination of selenium and bicalutamide produce a robust down-regulation of androgen receptor (AR) signaling, hTERT expression and telomerase activity. Furthermore, apoptosis induction by the two agents is more significant compared to single treatment. Our findings thus indicate that selenium in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of androgen deprivation therapy. Our data showed that selenium can induce DNA damage response in LNCaP cells. AR-mediated selenium suppression of hTERT expression is at transcriptional level. In addition, we found that wild-type and mutant AR response differentially to androgen under normoxia condition. However, in hypoxia condition, androgen up-regulates hTERT expression in both wild-type AR expression cells and mutant AR expressing cells. Selenium either alone or in combination with bicalutamide is able to inhibit androgen induced hTERT expression under hypoxia condition.					
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A. INTRODUCTION:

This project was designed to investigate telomerase as a potential target of androgen receptor (AR) signaling suppression by selenium. In the first annual report, we described that over-expression of hTERT attenuates the apoptosis inducing activities of selenium, supporting an important role of hTERT in selenium action in prostate cancer cells. Our data also provided an array of evidence supporting selenium in combination with an anti-androgen as a potential new modality for prostate cancer treatment. More importantly, we identified hTERT/telomerase as an important AR target mediating the bicalutamide/selenium effect. We also found that selenium can induce DNA damage response in LNCaP cells. In addition, our data showed that androgen-stimulated AR signaling induces the expression of hTERT through up-regulating hTERT promoter activity. In the second year period, we continued our study on the transcriptional regulation of hTERT by AR signaling, but shifted our research focus to the differential response of hTERT to androgen under normoxia vs. hypoxia condition.

B. BODY:

Aim 1: To assess the cellular mechanism by which hTERT/telomerase down-regulation mediates the anti-cancer effect of methylseleninic acid (MSA)

The effect of hTERT restoration on MSA-mediated growth inhibition.

Over-expression of hTERT weakens the apoptosis inducing activity of MSA in LNCaP cells. In order to study the biological significance of hTERT inhibition by MSA, we transiently transfected LNCaP cells with an hTERT expression construct, hTERT/pCI-Neo, and determined the effect of hTERT over-expression on MSA-induced apoptosis by using the Cell Death Detection ELISA^{PLUS} kit (Roche). As shown in Fig. 1, the effect of MSA on apoptosis induction is attenuated by hTERT over-expression.

Over-expression of hTERT does not affect MSA-mediated cell proliferation in LNCaP cells. We also investigated the effect of hTERT over-expression on cell proliferation by using the BrdU Cell Proliferation ELISA kit (Roche). As shown in Fig. 2, there is no difference in cell proliferation between mock-transfectant and hTERT-transfectant with MSA treatment, indicating that restoration of hTERT does not attenuate MSA effect on cell proliferation. The above data suggest that the mechanism by which hTERT/telomerase repression mediates MSA action is mainly through apoptosis induction.

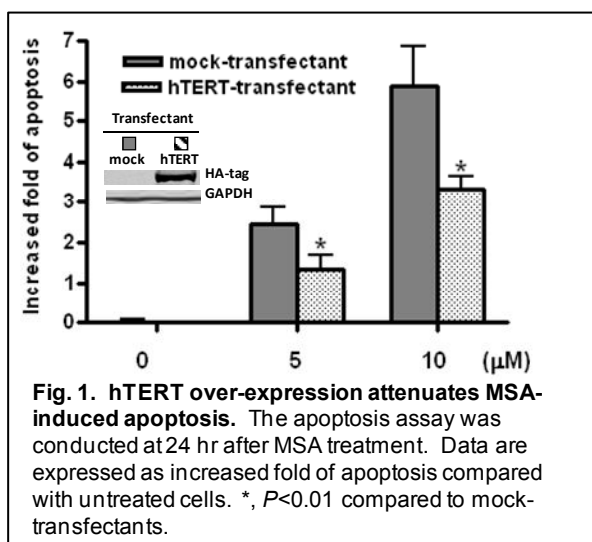


Fig. 1. hTERT over-expression attenuates MSA-induced apoptosis. The apoptosis assay was conducted at 24 hr after MSA treatment. Data are expressed as increased fold of apoptosis compared with untreated cells. *, $P < 0.01$ compared to mock-transfectants.

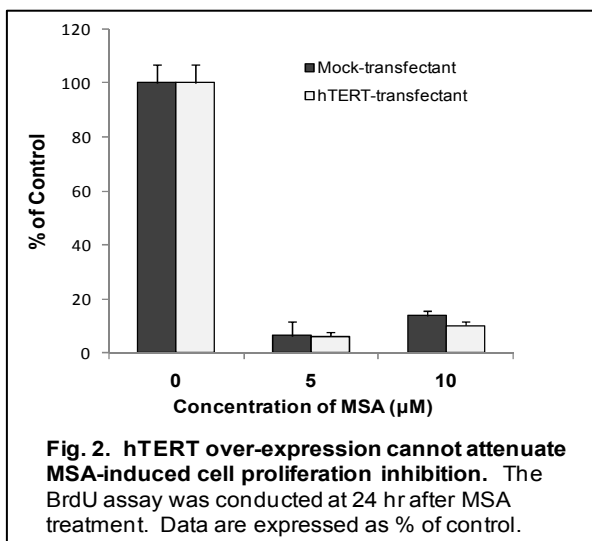
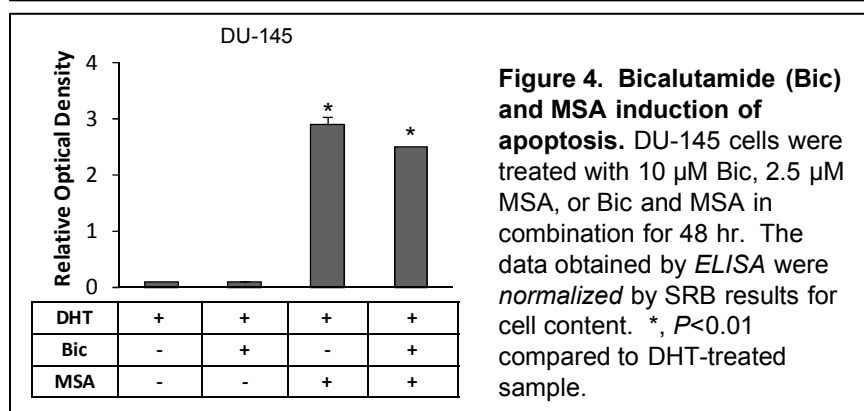
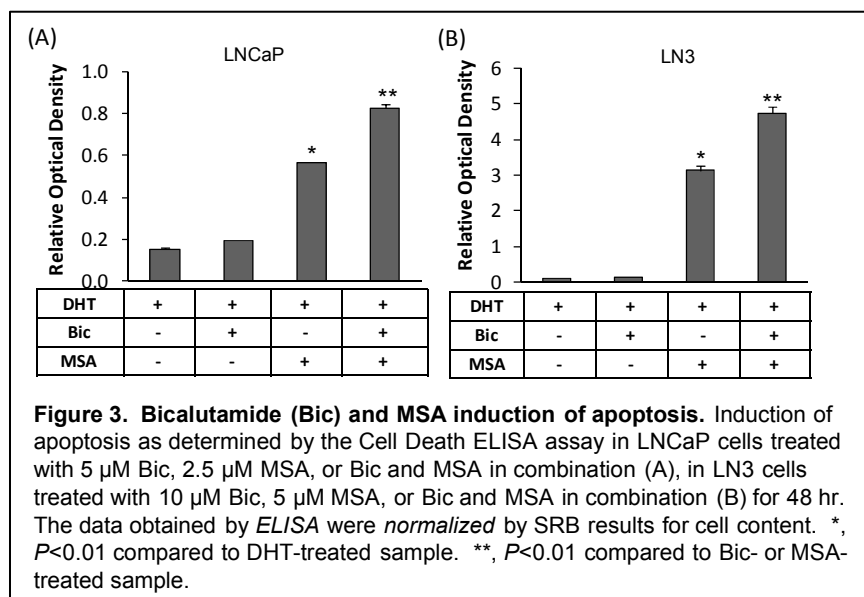


Fig. 2. hTERT over-expression cannot attenuate MSA-induced cell proliferation inhibition. The BrdU assay was conducted at 24 hr after MSA treatment. Data are expressed as % of control.



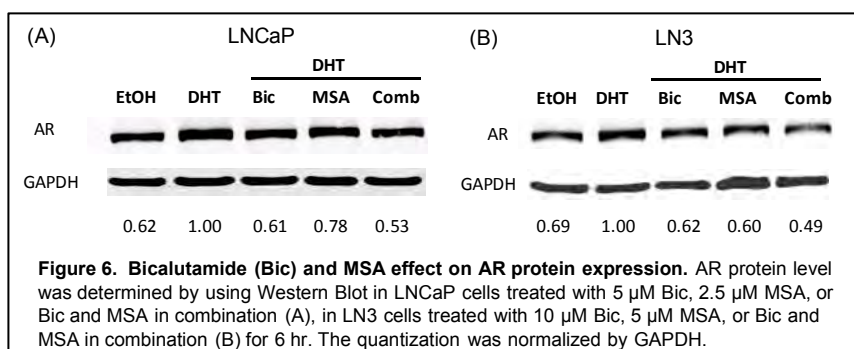
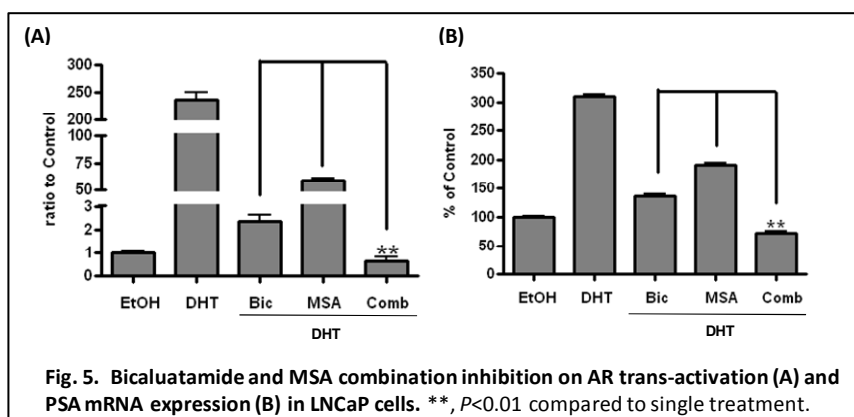
lead to further induction of apoptosis. The above data highly suggest that the induction of apoptosis by bicalutamide and MSA combination treatment is mainly through AR signaling pathway.

Combined bicalutamide and MSA treatment has greater suppression on AR signaling, thus leading to inhibition of hTERT expression and telomerase activity.

We then looked at the effect of bicalutamide and MSA combination on suppression of AR activity and protein expression. The luciferase assay shows that combination treatment can totally block DHT-induced AR transcriptional activity

Combined bicalutamide and MSA treatment has enhanced induction of apoptosis.

LNCaP and LN3 cells were treated with bicalutamide and/or MSA for 48 hr in the presence of dihydrotestosterone (DHT), and apoptosis was analyzed by using the Cell Death ELISA assay. As shown in Fig. 3, in both cell models, bicalutamide and MSA in combination led to a more striking increase of apoptosis than either agent alone. Conversely, the combination efficacy was not observed in the AR-negative DU-145 cell line (Fig. 4). In concordance with the previously-reported AR-independent activity of MSA in prostate cancer cells (1-5), MSA treatment of DU-145 cells resulted in a marked induction of apoptosis. However, the combination with bicalutamide was not able to

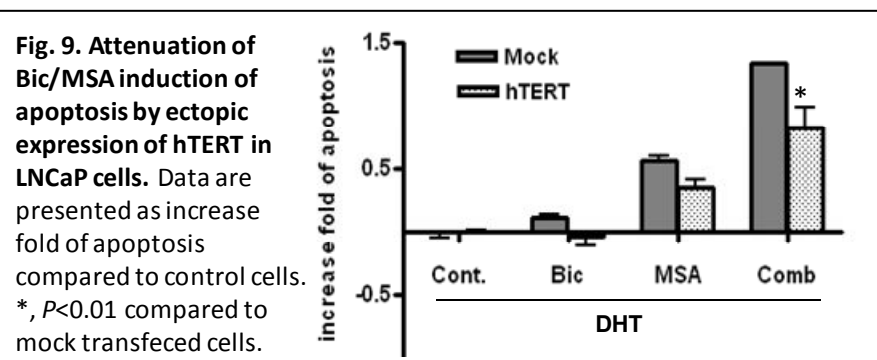
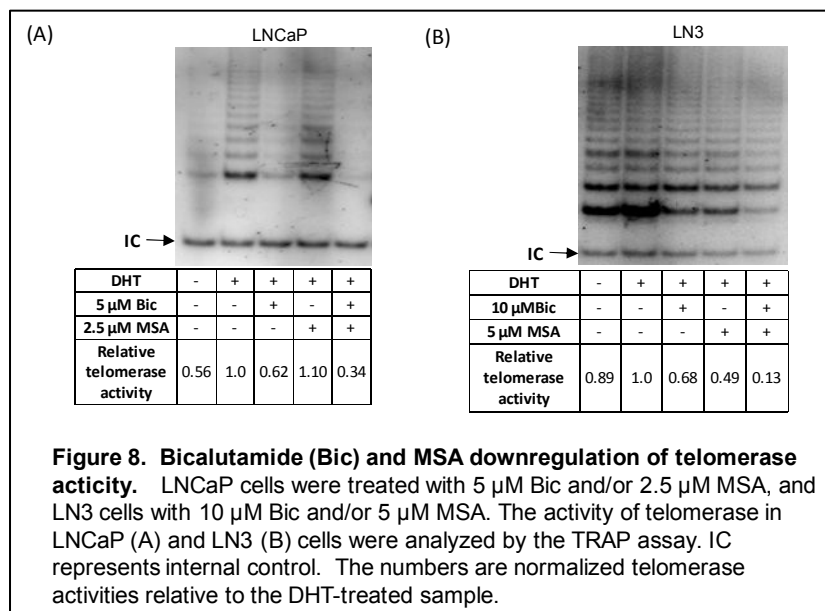
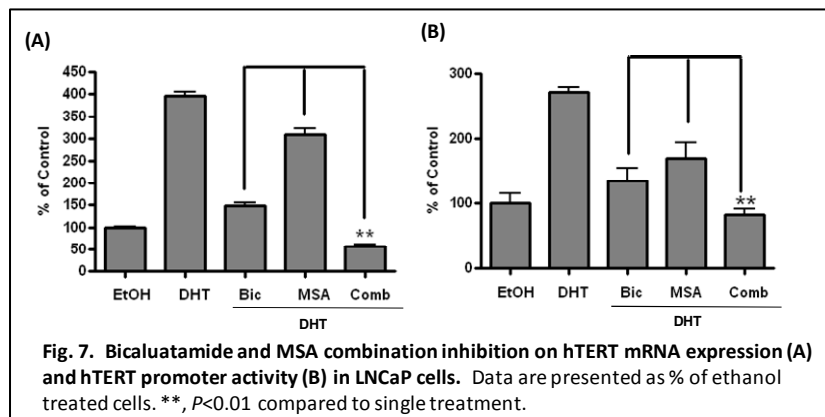


compared with single agents (Fig. 5A). This is also confirmed by our quantitative reverse transcription-PCR (qRT-PCR) data of prostate-specific antigen (PSA) mRNA expression. As shown in Fig. 5B, PSA expression is induced to 3-fold by DHT treatment. Bicalutamide and MSA can bring it down to ~1.4- or ~1.9-fold of vehicle control. The inhibition becomes more significant when these two agents are combined, which is only 70% of vehicle. Western blot result shows that in both LNCaP and LN3 cells, combination treatment could reduce more AR protein compared with single agents (Fig. 6). This is consistent with the repression of AR trans-activation (Fig. 5). The above data support our hypothesis that bicalutamide and MSA combination has better effect on AR signaling inhibition than single agents.

Next we proceeded to evaluate the effect of bicalutamide in combination with MSA on hTERT expression and telomerase activity. Fig. 7A shows that hTERT mRNA expression is induced to 4-fold with DHT treatment. Compared to bicalutamide and MSA treatment alone, combination treatment has the greatest suppression of hTERT expression (~58% of vehicle control). The hTERT promoter luciferase assay result shown in Fig. 7B is consistent with the mRNA data. As shown in Fig. 8A, telomerase activity in LNCaP cells was markedly induced by DHT. Bicalutamide or MSA alone produced minimal inhibition of DHT-induced telomerase activity. The two drugs in combination, however, almost completely blocked DHT induction of telomerase activity. The combination effect on telomerase activity was also manifested in LN3 cells (Fig. 8B). Thus, the telomerase activity data were qualitatively similar to the hTERT mRNA result (Fig. 7A).

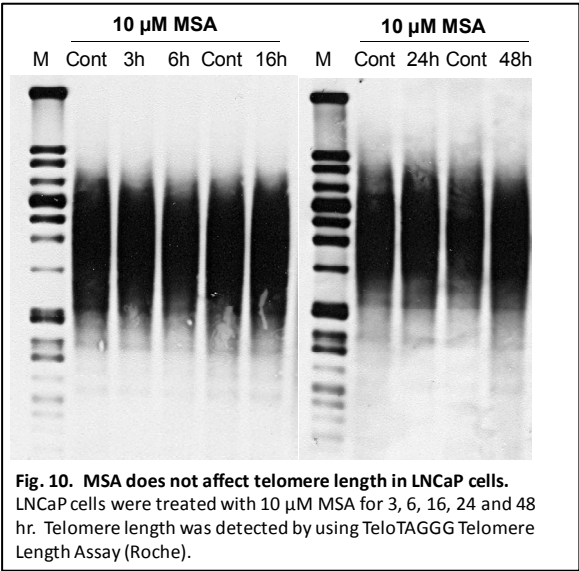
hTERT/telomerase is an important AR target mediating the bicalutamide/MSA effect.

In order to delineate the functional significance of hTERT downregulation in mediating the effect of bicalutamide and MSA, we transiently transfected LNCaP cells with an hTERT/pCI-Neo expression construct and assessed the response of the



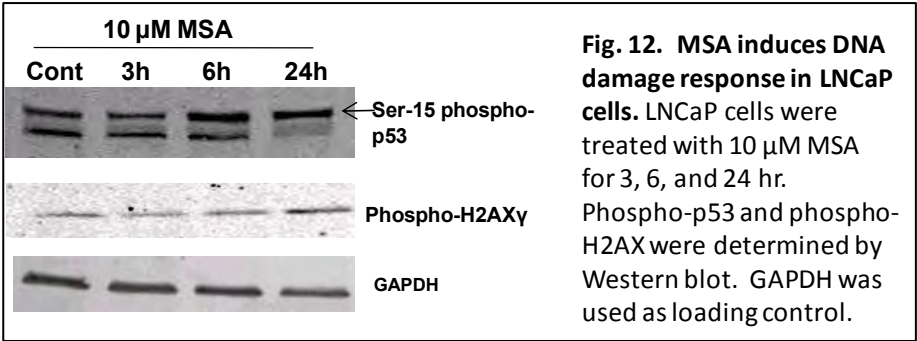
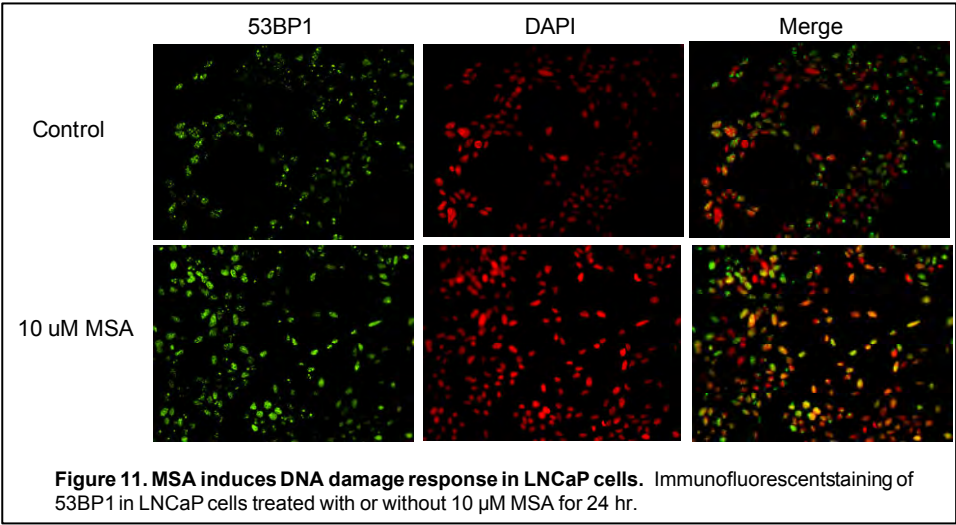
hTERT-overexpressing cells to the induction of apoptosis. As shown in Fig. 9, the restoration of hTERT not only weakened the apoptosis-inducing ability of bicalutamide or MSA alone, but also that of the combination, thus confirming the critical involvement of hTERT downregulation in mediating the combination effect.

Mechanism by which hTERT/telomerase suppression mediates the anti-cancer effect of MSA.



Our previous studies showed that 10 μM MSA induces a marked growth inhibition of prostate cancer cells at 48 hr. Telomerase suppression by MSA is unlikely to result in appreciable telomere shortening within such a short period of time. As expected we did not observed any change of telomere length up to 72-hour MSA treatment (Fig 10). Therefore, a mechanism independent of telomere shortening, such as telomere capping status, should be considered. Uncapped telomeres have been reported to trigger a rapid DNA damage response and lead to cell cycle arrest and/or apoptosis (6). As shown in Fig. 11, MSA induced more DNA damage foci than in control cells. To delineate these foci are located in telomere, we tried to co-stain 53BP1 with TRF1, which is a telomere associated protein used to indicating the

location of telomere. However, we still need to work on the quantization method of double staining method to show solid data. In addition, as shown in Fig. 12, 10 μM MSA induced phosphorylation of p53 and H2AX γ , confirming that MSA is able to induce DNA damage response in LNCaP cells.

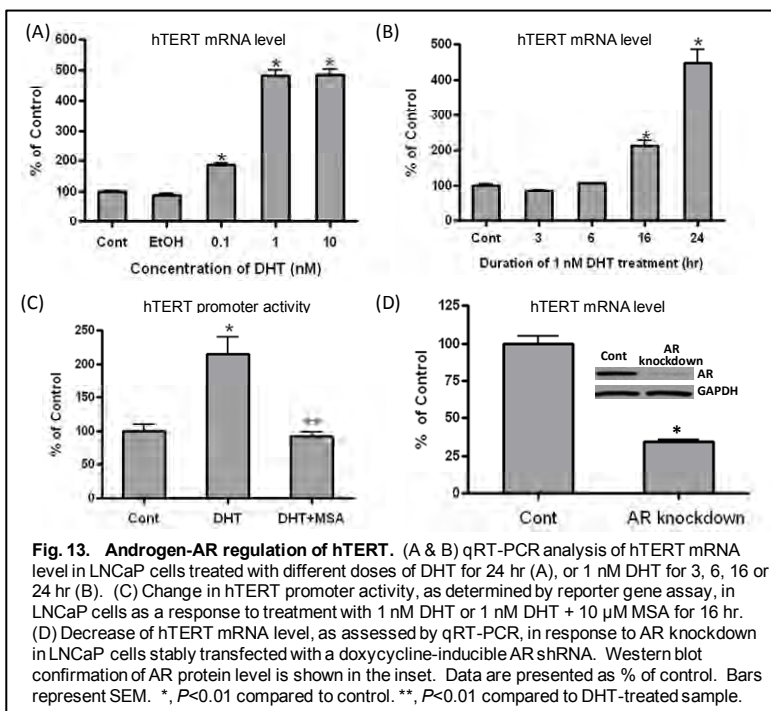


Aim 2: To study the mechanism by which AR signaling suppression contributes to the down-regulation of hTERT by MSA

Androgen signaling up-regulation of hTERT expression in LNCaP cells.

To study the effect of AR signaling on hTERT expression, qRT-PCR and luciferase assay were performed in LNCaP cells. As shown in Fig. 13 A&B, DHT can induce hTERT mRNA expression dose- and time- dependently. At 24 hr treatment, 0.1 nM DHT could induce hTERT mRNA to ~2-fold of control. With the dose increased to 1 nM, the induction became more dramatic (~5-fold). The magnitude remains at the same level with 10 nM DHT treatment. The obvious induction occurs at 16 hr and becomes more dramatic at 24 hr.

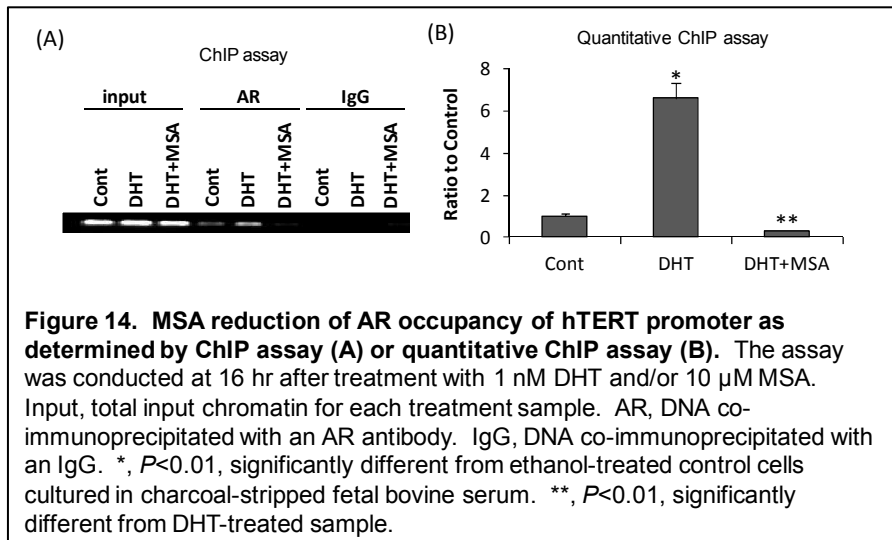
The luciferase assay result shows that the 4-kb hTERT promoter region is up-regulated by 1 nM DHT at 16 hr (Fig. 13C, the first two columns). The induction was almost completely blocked by 10 μ M MSA. We then studied the consequence of AR knockdown on hTERT expression. As shown in Fig. 13D, the knockdown of AR by treating LNCaP cells stably transfected with a doxycycline-inducible AR-shRNA lentiviral system with doxycycline leads to a significant reduction of hTERT mRNA. The data therefore suggest that suppression of AR signaling by either MSA treatment or AR knockdown could efficiently inhibit hTERT expression.



Selenium reduces AR recruitment to hTERT promoter region.

In order to demonstrate that suppressed hTERT promoter activity is attributable to reduced AR occupancy of the hTERT promoter as a consequence of MSA downregulation of AR, we performed the Chromatin

Immunoprecipitation (ChIP) assay on the hTERT promoter region that was reported to associate with AR (7). Fig. 14A shows the result of PCR amplification of DNA co-immunoprecipitated with either an AR antibody or IgG using primers specific for the hTERT promoter region. The specificity of the ChIP assay is evident from the difference in band



intensity between the AR-antibody-immunoprecipitated samples and the IgG-immunoprecipitated samples. In concordance with a previous report (7), AR is only weakly associated with the hTERT promoter in the absence of androgen in LNCaP cells. DHT significantly increased AR occupancy of the promoter, and the increase was blocked by 10 μ M MSA. We then conducted qRT-PCR of the co-immunoprecipitated DNA to quantify the change in AR occupancy of hTERT promoter in response to different treatments. As shown in Fig. 14B, DHT treatment induced a ~6-fold increase of AR occupancy of the promoter, and the increase was completely suppressed by MSA.

LNCaP and LAPC-4 cells respond differentially to

DHT treatment. In addition to LNCaP cells, we also investigated androgen effect on hTERT expression in another androgen-dependent cell line, LAPC-4 which expresses wild-type AR. Interestingly, we found that in contrast to up-regulation of hTERT expression in LNCaP cells, DHT actually inhibits hTERT mRNA expression in LAPC-4 cells (Fig. 15). As approved by the DOD Prostate Cancer Research Program, we shifted our research focus to the differential response of hTERT to androgen under normoxia vs. hypoxia condition in order to investigate why wild-type and mutant AR respond differentially to androgen treatment regarding hTERT expression.

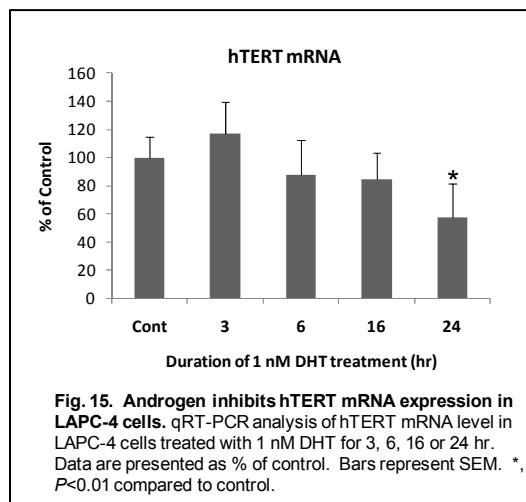


Fig. 15. Androgen inhibits hTERT mRNA expression in LAPC-4 cells. qRT-PCR analysis of hTERT mRNA level in LAPC-4 cells treated with 1 nM DHT for 3, 6, 16 or 24 hr. Data are presented as % of control. Bars represent SEM. *, $P < 0.01$ compared to control.

Androgen signaling regulation of hTERT expression under normoxia and hypoxia conditions.

LNCaP cells were cultured with androgen-deprived medium under both normoxia and hypoxia condition for 24 hr before 1 nM DHT treatment for 24 hr. qRT-PCR was performed to determine the hTERT mRNA level. As shown in Fig. 16A, at 24 hr treatment, 1 nM DHT could induce hTERT mRNA to ~4-fold of control under normoxia condition. The induction became more dramatic (~6-fold) when cells were cultured in hypoxia condition. We then compared the DHT effect on hTERT expression in LAPC-4 cells under normoxia vs. hypoxia condition. As shown in Fig. 16B, 1 nM DHT inhibited hTERT mRNA expression to ~50% of control under normoxia condition. However, DHT actually up-regulated hTERT mRNA level to ~3 folds of vehicle treated control when LAPC-4 cells were cultured in hypoxia condition. The opposite effect of androgen on hTERT expression indicates that under hypoxia condition which is much similar to tumor environment, there might be some molecules or pathways that could facilitate wild-type AR to activate hTERT expression.

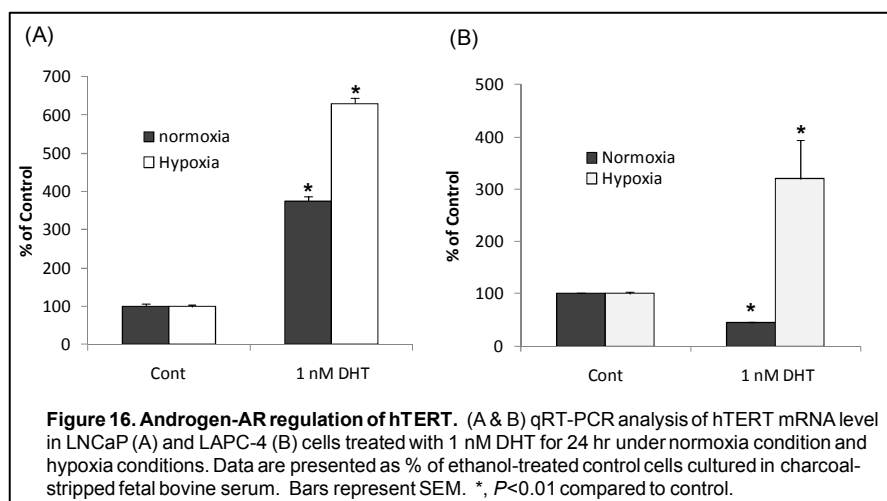


Figure 16. Androgen-AR regulation of hTERT. (A & B) qRT-PCR analysis of hTERT mRNA level in LNCaP (A) and LAPC-4 (B) cells treated with 1 nM DHT for 24 hr under normoxia condition and hypoxia conditions. Data are presented as % of ethanol-treated control cells cultured in charcoal-stripped fetal bovine serum. Bars represent SEM. *, $P < 0.01$ compared to control.

Selenium inhibits hTERT expression under hypoxia condition in both LNCaP and LAPC-4 cells.

We next examined the effect of MSA on hTERT expression under hypoxia condition in LNCaP and LAPC-4 cells. As shown in Fig. 17A, in LNCaP cells, DHT could induce hTERT mRNA expression to ~ 6 folds; this induction was almost blocked by MSA treatment. We observed the same result in LAPC-4 cells that MSA could also inhibit DHT induced hTERT expression (Fig. 17B). Therefore, our data indicate that MSA has the ability to repress hTERT expression regardless of the culture condition.

We also investigate the effect of bicalutamide in combination with MSA on hTERT expression under hypoxia condition. As shown in Fig. 18, in LNCaP cells we observed the similar combinatorial effect on hTERT suppression as in normoxia culture condition (Fig. 7B).

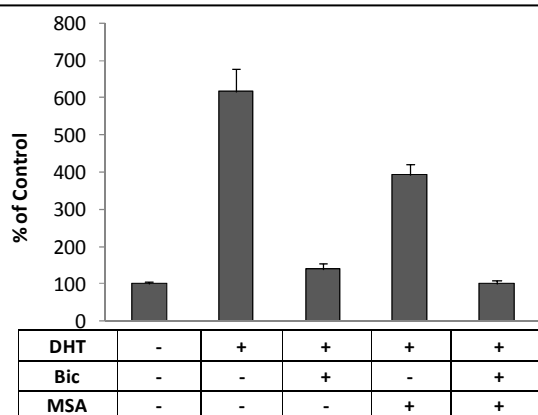
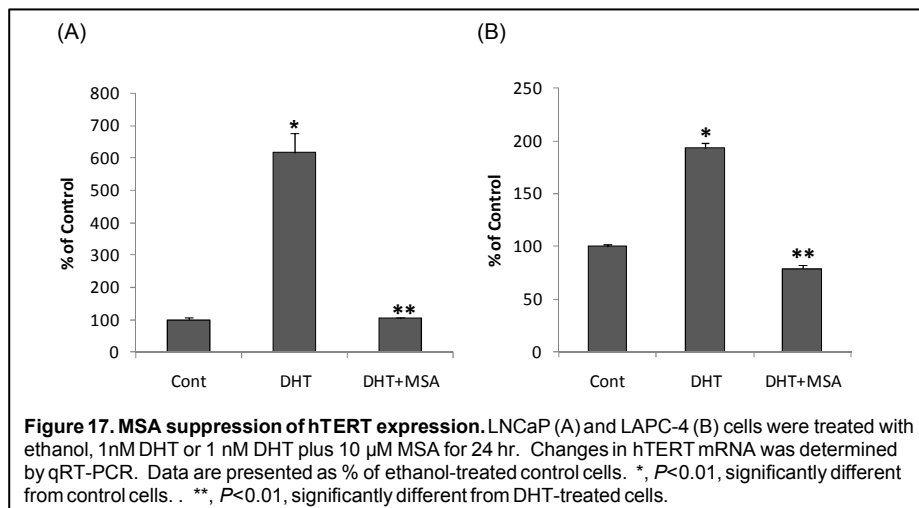


Fig. 18. Combinatorial effect of bicalutamide and MSA on hTERT expression in LNCaP cells cultured under hypoxia condition.

C. KEY RESEARCH ACCOMPLISHMENTS:

- Over-expression of hTERT attenuates the apoptosis induction activity of selenium in LNCaP cells.
- Bicalutamide and selenium combination has greater apoptosis induction in AR-positive cells rather than AR-negative cells compared to single agents.
- Bicalutamide and selenium combination has greater suppression on AR signaling, thus leading to inhibition of hTERT expression and telomerase activity.
- hTERT/telomerase is an important AR target mediating the bicalutamide/selenium effect.
- Selenium reduces AR recruitment to hTERT promoter region.
- In addition to AR protein level, other mechanism(s) might participate in selenium repression of hTERT.
- AR signaling regulates hTERT expression at transcriptional level.
- Wild-type and mutant AR have different effect on hTERT mRNA expression.
- In normoxia and hypoxia conditions, DHT has different effect on hTERT mRNA expression in LAPC-4 cells expressing wild-type AR.
- Selenium either alone or in combination with bicalutamide inhibits hTERT expression under both normoxia and hypoxia condition.

D. REPORTABLE OUTCOMES:

Publication(s):

- Liu S, Qi Y, Ge Y, Duplessis T, Rowan BG, Ip C, Cheng H, Rennie PS, Horikawa I, Lustig A, Yu Q, Zhang H, and Dong Y. Telomerase as an important target of androgen-signaling blockade for prostate cancer treatment. Mol Cancer Ther 2010 Jul; 9(7):2016-25.
(Highlights: Selected article from this issue)

Presentation(s):

- 101st American Association for Cancer Research (AACR) Annual Meeting, 2010 April, “Telomerase as an important target of androgen-signaling blockade for prostate cancer treatment” (poster presentation)
- 20th Annual Tulane Health Sciences Research Day, 2008, “Down-regulation of telomerase by selenium in prostate cancer cells” (poster presentation)
- The Breast Ovarian Cancer Journal Club/Seminar Series, 2008, “Down-regulation of telomerase by selenium in prostate cancer cells” (oral presentation)

E. CONCLUSIONS:

The result from our current study suggests that hTERT, a novel target of AR signaling, plays an important role in mediating selenium action in human prostate cancer cells. Our data also provided an array of evidence supporting selenium in combination with bicalutamide, an anti-androgen, as a potential new modality for not only the prevention but also the treatment of prostate cancer. More importantly, the identification of hTERT/telomerase as an important AR target mediating the bicalutamide/MSA effect has great clinical implications. Telomerase activation has been reported in >90% of prostate cancer samples, but not in normal or benign prostatic hyperplasia tissues.

Telomerase activation has been well documented to play an essential role in cell survival and oncogenesis, and inhibition of telomerase has been shown to suppress growth and tumorigenic potential of prostate cancer cells. Blocking telomerase activation by anti-androgen and MSA through suppressing AR signaling could thus represent an effective and selective treatment modality to target prostate cancer cells. In addition, hTERT/telomerase could be measured in blood and urine and, therefore, could serve as a noninvasive, tumor-specific, functionally relevant molecular biomarker for monitoring the efficacy of the intervention. We also elucidated that the repression of hTERT by selenium is through inhibition of AR recruitment to hTERT promoter region, thus down-regulation of hTERT transcription. In addition, we demonstrated that wild-type and mutant AR have differential response to androgen; wild-type AR has different response to androgen treatment under normoxia and hypoxia condition. Selenium either alone or combined with bicalutamide could inhibit hTERT expression in hypoxia condition. Our current finding not only broadens our understanding of the mechanism of selenium action but also has great clinical implications.

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APPENDICES

Telomerase as an important target of androgen-signaling blockade for prostate cancer treatment

Shuang Liu¹, Yanfeng Qi¹, Yubin Ge³, Tamika Duplessis¹, Brian G. Rowan¹, Helen Cheng⁴, Paul S. Rennie⁴, Haitao Zhang², and Yan Dong¹

¹*Departments of Structural and Cellular Biology, ²Pathology and Laboratory Medicine, Tulane University School of Medicine, Tulane Cancer Center, New Orleans, LA 70112; ³Developmental Therapeutics Program, Karmanos Cancer Institute, and Department of Pediatrics, Wayne State University School of Medicine, Detroit, MI 48201; ⁴the Vancouver Prostate Centre, Vancouver, BC Canada V6H 3Z6*

As the mainstay treatment for advanced prostate cancer, androgen-deprivation therapy (ADT) targets the action of androgen receptor (AR) by reducing androgen level and/or by the administration of anti-androgen that competes with androgens for binding to AR. Albeit effective in extending survival, ADT is associated with dose-limiting toxicity and the development of castration-resistant prostate cancer (CRPC) after prolonged use. Since CRPC is generally lethal and incurable, developing effective strategies to enhance the efficacy of ADT and circumvent resistance becomes an urgent task. Continuous AR signaling constitutes one major mechanism underlying the development of CRPC. The present study showed that methylseleninic acid (MSA), an agent that effectively reduces AR abundance, could enhance the cancer-killing efficacy of the anti-androgen bicalutamide in both androgen-dependent and castration-resistant prostate cancer cells. We found that combination of MSA and bicalutamide produced a robust downregulation of prostate-specific antigen and a recently identified AR target, telomerase and its catalytic subunit, telomere reverse transcriptase (hTERT). The downregulation of hTERT occurs mainly at the transcriptional level, through reducing AR occupancy of the hTERT promoter. Furthermore, apoptosis induction by the two agents is significantly mitigated by restoration of hTERT. Our findings thus indicate that MSA in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of ADT. Given the critical role of hTERT/telomerase downregulation in mediating the combination effect and the fact that hTERT/telomerase could be measured in blood and urine, hTERT/telomerase could serve as an ideal tumor-specific biomarker to monitor the efficacy of the combination therapy non-invasively. (Supported by the Department of Defense Prostate Cancer Training grant No. W81XWH-08-1-0291 (SL); the National Cancer Institute grant No. K01 CA114252 (YD); the American Cancer Society grant No. RSG-07-218-01-TBE (YD))

Down-regulation of telomerase by selenium in prostate cancer cells

Shuang Liu and Yan Dong

Department of Structural and Cellular Biology, Tulane University Health Science Center, New Orleans, LA 70112

The Nutritional Prevention of Cancer Trial showed that selenium supplementation reduced prostate cancer incidence by ~50%, although the underlying mechanism remains unclear. Telomerase activation is a rate-limiting step in cellular immortalization and oncogenesis. In the present study, we demonstrated a dose- and time-dependent drop in telomerase activity as a result of selenium treatment in human prostate cancer cells. The reduction was mostly attributable to a significant decrease in the level of the catalytic subunit, human telomerase reverse transcriptase (hTERT), the major determinant of telomerase enzymatic activity. Such effect of selenium was detected in 4/4 of the androgen-dependent and –independent human prostate cancer cell lines examined, suggesting the universality of the phenomenon. Results from mRNA stability analysis and nuclear run-on assay indicated hTERT downregulation occurred mainly at the transcriptional level. The expression of the hTERT gene is known to be regulated by a number of transcription factors, including androgen receptor (AR). AR plays an important role in the development and progression of prostate cancer, and selenium treatment led to a marked decrease in the expression and *trans*-activating/DNA-binding activity of AR. We therefore investigated the potential involvement of AR in selenium downregulation of hTERT. We found that activation of AR signaling by dihydrotestosterone (DHT) increased hTERT mRNA transcription. This effect was both dose- and time-dependent. On the other hand, AR knockdown decreased hTERT mRNA expression and enhanced the suppression effect of selenium on hTERT. However, restoration of AR by overexpression was not able to reverse selenium effect on hTERT, suggesting the involvement of mechanisms in addition to decreasing AR abundance, such as disrupting the interaction between AR and AR co-regulators. We are currently using ChIP assay to study the recruitment of AR to the hTERT promoter under selenium treatment. Telomerase activation has been detected in the vast majority of tumor samples, and is one of the most widespread tumor markers. In prostate cancer, the activation is already evident at early stages of the disease. Consequently, telomerase represents an attractive target for prostate cancer prevention and treatment. Our novel findings therefore provided justification for a mechanism-driven strategy in using selenium to control prostate cancer development and progression. In addition, considering the fact that telomerase/hTERT could be measured in the circulation, our data could lead to the identification of a new non-invasive molecular biomarker for future selenium intervention trials to gauge the efficacy of intervention. (Supported by NCI grant CA114252 and ACSRSG-07-218-01-TBE).

Metronomic Gemcitabine Inhibits Pancreatic Cancer

Tran Cao *et al.* _____ Page 2068

Tran Cao and colleagues evaluated the efficacy of metronomic gemcitabine against metastasis formation in a highly-aggressive, RFP-labeled orthotopic nude mouse model of human pancreatic cancer. Metronomic gemcitabine reduced the spontaneous development of both solid metastases and ascites and significantly prolonged survival of mice without overt toxicity. These results suggest the clinical potential of adjuvant metronomic gemcitabine for the treatment of pancreatic cancer.

MK-2206 Sensitizes Tumors to Chemotherapy

Hirai *et al.* _____ Page 1956

Akt lies at a critical signaling node downstream of PI3K and is important in promoting cell survival and inhibiting apoptosis. Increased Akt signaling is associated with reduced sensitivity to cytotoxic agents or receptor tyrosine kinase inhibitors in preclinical models. The effect of a novel allosteric Akt inhibitor, MK-2206, was evaluated in combination with several anticancer agents. MK-2206 showed synergistic antitumor activities in combination with erlotinib, lapatinib, and cytotoxic agents, suggesting Akt inhibition may augment the efficacy of anticancer agents. MK-2206 (now in Phase I) is a promising agent to treat cancer patients who receive these cytotoxic and/or molecular targeted agents.

SCC Apoptosis Induction by PS-341 and HDAC Inhibitors

Kim *et al.* _____ Page 1977

Head and neck squamous cell carcinoma (HNSCC) is relatively resistant to chemotherapy-mediated apoptosis and frequently develops chemoresistance. Thus, improvement on conventional therapy is urgently needed to effectively treat HNSCC. In this study, Kim and colleagues found that the histone deacetylase inhibitor trichostatin A (TSA) significantly enhanced apoptosis in HNSCC induced by PS-341 (also known as bortezomib) *in vitro* and improved PS-341-mediated inhibition of HNSCC tumor growth in nude mice. Mechanistically, TSA increased PS-341-induced Noxa expression and caspase activation in HNSCC cells. These results provide an important rationale for the usage of a combination of both agents in patients with HNSCC.

Methylseleninic Acid Enhances Anti-Androgen Efficacy

Liu *et al.* _____ Page 2016

The development of castration-resistant prostate cancer after androgen deprivation therapy remains the major challenge in the treatment of advanced prostate cancer. Liu and colleagues showed that methylseleninic acid, an agent that effectively reduces androgen receptor abundance, significantly enhanced the cancer-killing efficacy of anti-androgen. Downregulation of telomerase as a result of androgen receptor signaling suppression is critically involved in mediating the combination effect. The findings indicate that methylseleninic acid in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of androgen deprivation therapy and that telomerase could serve as a tumor-specific biomarker to monitor the efficacy.

Telomerase as an Important Target of Androgen Signaling Blockade for Prostate Cancer Treatment

Shuang Liu¹, Yanfeng Qi¹, Yubin Ge⁴, Tamika Duplessis¹, Brian G. Rowan¹, Clement Ip⁵, Helen Cheng⁶, Paul S. Rennie⁶, Izumi Horikawa⁷, Arthur J. Lustig², Qun Yu⁸, Haitao Zhang^{3,9}, and Yan Dong^{1,9}

Abstract

As the mainstay treatment for advanced prostate cancer, androgen deprivation therapy (ADT) targets the action of androgen receptor (AR) by reducing androgen level and/or by using anti-androgen to compete with androgens for binding to AR. Albeit effective in extending survival, ADT is associated with dose-limiting toxicity and the development of castration-resistant prostate cancer (CRPC) after prolonged use. Because CRPC is lethal and incurable, developing effective strategies to enhance the efficacy of ADT and circumvent resistance becomes an urgent task. Continuous AR signaling constitutes one major mechanism underlying the development of CRPC. The present study showed that methylseleninic acid (MSA), an agent that effectively reduces AR abundance, could enhance the cancer-killing efficacy of the anti-androgen bicalutamide in androgen-dependent and CRPC cells. We found that the combination of MSA and bicalutamide produced a robust downregulation of *prostate-specific antigen* and a recently identified AR target, telomerase, and its catalytic subunit, *human telomerase reverse transcriptase*. The downregulation of *hTERT* occurs mainly at the transcriptional level, and reduced AR occupancy of the promoter contributes to downregulation. Furthermore, apoptosis induction by the two agents is significantly mitigated by the restoration of *hTERT*. Our findings thus indicate that MSA in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of ADT. Given the critical role of *hTERT*/telomerase downregulation in mediating the combination effect and the fact that *hTERT*/telomerase could be measured in blood and urine, *hTERT*/telomerase could serve as an ideal tumor-specific biomarker to monitor the efficacy of the combination therapy noninvasively. *Mol Cancer Ther*; 9(7); 2016–25. ©2010 AACR.

Introduction

Telomerase is a ribonucleoprotein enzyme charged with attaching telomeric DNA to the ends of chromosomes and, thus, preventing chromosomes from degradation and end-to-end fusion (1). The role of telomerase in tumorigenesis has been well documented. It is activated in 85% to

90% of malignant tumors, but generally not in normal somatic cells (2). The reactivation of telomerase allows cells to overcome replicative senescence, thereby leading to cellular immortalization and oncogenesis. Inhibition of telomerase has been shown to limit life span, impair cell growth, and suppress the tumorigenic potential of cancer cells of different organs, including prostate (3, 4).

Human telomerase consists of a telomerase RNA component, a telomere reverse transcriptase (*hTERT*), and telomerase-associated proteins (2). The telomerase RNA component and telomerase-associated proteins are constitutively expressed in cancer and normal cells, irrespective of the presence or absence of telomerase activity. However, a strong correlation has been observed between *hTERT* mRNA expression and telomerase activity (5, 6).

The expression of *hTERT* and the activity of telomerase are regulated by androgen receptor (AR) signaling. In LNCaP human prostate cancer cells that express a mutant but functional AR, androgen was shown to induce telomerase activity by recruiting AR to the *hTERT* promoter and increasing its transcription (7, 8). However, exogenously expressed wild-type AR was shown to inhibit *hTERT* transcription in an AR-null prostate cancer cell line (8). The data, nonetheless, cannot be generalized to the conclusion that wild-type suppresses *hTERT* expression. Activated AR is known to inhibit the growth of

Authors' Affiliations: Departments of ¹Structural and Cellular Biology, ²Biochemistry, and ³Pathology and Laboratory Medicine, Tulane University School of Medicine, Tulane Cancer Center, New Orleans, Louisiana; ⁴Developmental Therapeutics Program, Karmanos Cancer Institute, and Department of Pediatrics, Wayne State University School of Medicine, Detroit, Michigan; ⁵Roswell Park Cancer Institute, Buffalo, New York; ⁶The Vancouver Prostate Centre, Vancouver, British Columbia, Canada; ⁷The National Cancer Institute, Bethesda, Maryland; ⁸Beijing Institute of Transfusion Medicine, Beijing, China; and ⁹School of Basic Medicine, Jilin University, Changchun, Jilin, China

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Corresponding Authors: Yan Dong, Department of Structural and Cellular Biology, Tulane University School of Medicine, 1430 Tulane Avenue SL-49, New Orleans, LA 70112. Phone: 504-988-4761; Fax: 504-988-0468. E-mail: ydong@tulane.edu; Haitao Zhang, Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, LA 70112. E-mail: hzhang@tulane.edu; and Qun Yu, Beijing Institute of Transfusion Medicine, Beijing 100850, China. E-mail: yuq@nic.bmi.ac.cn

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AR-null cells, as opposed to the growth-stimulatory effect seen in prostate cancer cells expressing endogenous AR (9). In fact, evidence from both xenograft and human prostate cancer studies have indicated that endogenously expressed wild-type AR positively regulates *hTERT* expression (7, 10). Castration was shown to decrease *hTERT* expression and telomerase activity in CWR22 xenograft tumors (7), which express a wild-type AR (11), and these effects were reversed by the subsequent administration of androgen (7). A study of 30 prostate cancer patients underwent androgen deprivation therapy (ADT) before prostatectomy showed that *hTERT* expression dropped to 36% of pretreatment level as a result of ADT (10). AR genotyping was not done in this study. However, because AR mutations occur at low levels in prostate carcinomas (12), it is reasonable to believe that the majority of the prostate cancer cells in the patients express a wild-type AR. Collectively, the data show a positive regulation of *hTERT*/telomerase by endogenously expressed AR in prostate tumors, and suppression of AR signaling could represent a viable approach for blocking telomerase activation in the tumor.

Our previous studies showed that methylated forms of selenium are very effective in reducing AR abundance and thereby AR *trans*-activation (13–16). Selenium is an effective anticancer agent in cancers of various sites, including the prostate, by numerous preclinical studies, epidemiologic observations, and clinical trials (17, 18). Although the interim analysis of the Selenium and Vitamin E Chemoprevention Trial (SELECT) indicated that supplementation of healthy individuals with nutritional dose of selenium did not reduce prostate cancer risk (19), the finding should not have been entirely unexpected. The anticancer efficacy of selenium depends on the form and dosage of selenium administered and the stage of the disease progression (17, 20–23). In fact, animal studies using xenograft models have shown that selenomethionine, the form of selenium used in the SELECT, is ineffective against prostate cancer growth, whereas “second generation” selenium compounds, including methylseleninic acid (MSA), are very effective in inhibiting tumor growth (21, 23). This is in concordance with the fact that selenomethionine is converted to the active metabolite, methylselenol, much less efficiently than the much more potent new selenium compounds (17, 20, 24). Additionally, in most of the preclinical studies that showed a positive association between selenium administration and tumor inhibition, the experiments were conducted by using pharmacologic doses of selenium, not the nutritional dose that was used in the SELECT. Therefore, the negative SELECT finding underscores the urgency of studying the efficacy of the new selenium compounds, particularly at pharmacologic doses, for prostate cancer intervention. Selenium has a low toxicity profile. Although the recommended dietary allowance and the tolerable upper intake level for selenium for adults are set at 55 and 400 $\mu\text{g}/\text{d}$, respectively (25), patients with biopsy-proven prostate cancer supplemented

with 3,200 μg selenium per day for an average of 1 year did not develop any serious selenium-related toxicities (26). Therefore, selenium supplementation at pharmacologic doses will be well tolerated.

We have previously reported that treatment of prostate cancer cells with MSA, at a concentration between 2.5 and 10 $\mu\text{mol}/\text{L}$, could markedly decrease the levels of AR mRNA and protein (13, 14). In mice given a nontoxic dose of MSA, serum selenium level reaches a peak level of 12.5 $\mu\text{mol}/\text{L}$ at 1 hour and gradually declines to basal level by 24 hours (27). Therefore, the concentrations of MSA that could suppress AR expression are physiologically relevant. We have also found that the reduced abundance of AR results in downregulated AR *trans*-activation. Ectopic expression of AR reverses MSA suppression of both AR *trans*-activation and cell growth, suggesting that AR signaling is a key mediator of MSA effect (14). The findings lend credence to the idea that MSA should also be effective in suppressing androgen-induced *hTERT* expression and telomerase activity. In addition, because MSA and anti-androgen target AR signaling at different levels, through reducing receptor availability and disrupting ligand binding, respectively, combining these two drugs would be expected to produce a more pronounced suppression of AR *trans*-activation and subsequently *hTERT*/telomerase, thereby inhibiting the growth/survival of prostate cancer cells. Therefore, the present study was designed to determine the potential of using MSA to increase the efficacy of anti-androgen by characterizing the effects of MSA and an anti-androgen, bicalutamide (Casodex), either alone or in combination, on *hTERT* expression, telomerase activation, and apoptosis in androgen-dependent and castration-resistant prostate cancer (CRPC) cells.

Materials and Methods

Prostate cancer cell lines and reagents

The LNCaP, 22Rv1, and DU-145 human prostate cancer cell lines were obtained from the American Type Culture Collection at passage 4. Cells used in this study were within 20 passages (~ 3 mo of noncontinuous culturing). The LAPC-4 cell line (28) was provided by Dr. Charles L. Sawyers at the University of California at Los Angeles Jonsson Comprehensive Cancer Center, Los Angeles, CA. The two castration-resistant LNCaP sublines, LN3 (29) and C81 (30), were obtained from Dr. Curtis A. Pettaway (University of Texas M.D. Anderson Cancer Center, Houston, TX) and Dr. Ming-Fong Lin (University of Nebraska Medical Center, Omaha, NE), respectively. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 U/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin. In some experiments, cells were cultured in an androgen-defined condition by using 10% charcoal-stripped FBS in the absence or presence of dihydrotestosterone (DHT). MSA and bicalutamide were purchased from PharmaSe and Toronto Research Chemicals, Inc., respectively.

Apoptosis detection

Detached cells were precipitated by centrifugation and pooled with attached cells. Cytoplasmic histone-associated DNA fragments were quantified by using the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science) as per the manufacturer's protocol. The absorbance was measured at 405 nm (reference wavelength at 490 nm) and normalized by the SRB value for cell content.

Quantitative reverse transcription-PCR

The analysis was done as previously described (13) using RNA isolated with the PerfectPure RNA Cultured Cell kit (Fisher Scientific, LLC). The PCR primers and Taqman probes for *β-actin* (a housekeeping gene), *prostate-specific antigen (PSA)*, and *hTERT* were products of Applied Biosystems.

Western blot analysis

Details of the procedure for Western blot analysis were previously described (31). Immunoreactive bands were quantitated by volume densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase. The following antibodies were used in this study (source): anti-glyceraldehyde-3-phosphate dehydrogenase (Millipore), anti-AR (Millipore), and anti-HA (Covance, Inc.).

Telomerase activity assay

Telomerase activity was analyzed by using the TRAPeze Telomerase Detection kit (Millipore) per the manufacturer's protocol with minor modification. In brief, 2×10^5 cells were lysed in 200 μ L of CHAPS lysis buffer. Protein concentration of the lysate was determined with the bicinchoninic acid protein assay kit (Pierce), and an amount of 1.5 μ g was used in the PCR reaction. The extract was incubated at 30°C for 60 minutes in 50 μ L of reaction solution containing the TS primer, reverse primer, deoxynucleotide triphosphates, TRAP reaction buffer (provided by the kit), and Titanium Taq DNA polymerase (Clontech). The products were then subjected to PCR amplification with one cycle at 94°C for 2 minutes and followed by 29 cycles at 94°C for 30 seconds and 59°C for 45 seconds. A pair of internal-control primers was included to monitor the amplification efficiency of each PCR reaction. For each sample, a heat inactivation telomerase control was tested to ensure the specificity of the reaction. The PCR products were separated on a 28 $\times$ 20 cm 12% nondenaturing polyacrylamide gel and visualized by SYBR Green staining. The signal of the TRAP product ladder bands for each sample was quantitated by volume densitometry using the NIH ImageJ software, and normalized to that of the internal control band.

AR knockdown

LNCaP cells stably transfected with a doxycycline-inducible AR-shRNA lentiviral system was generated as previously described (32). Doxycycline at a concentration of 2 μ g/mL was added to the cells 24 hours before

the addition of MSA to induce AR knockdown. The MSA treatment continued for 16 hours.

Transient transfection of an hTERT expression vector

The hTERT expression vector, hTERT/pCI-Neo, was purchased from Addgene (# Plasmid 1782). The transfection was carried out in a 10-cm culture dish as previously described (33), and the cells were subsequently trypsinized and replated in triplicate onto a 96-well plate for apoptosis analysis. Cells were allowed to recover for an additional 48 hours before treatment.

Reporter gene assay

The hTERT promoter construct, hTERT-3915/pGL4, was generated by subcloning a ~4-kb (–3,915 to +40) hTERT promoter region from the pBT-3915 plasmid (34) into the pGL4.19 (luc2CP/neo) rapid response luciferase expression vector (Promega). Transfection and luciferase activity analyses were done as previously described (33). To obtain uniform transfection efficiency, transfection was conducted in a 10-cm culture dish, and the cells were subsequently trypsinized and replated in triplicate onto six-well plates for treatments. Luciferase activity was normalized by the protein concentration of the sample.

mRNA stability assay

Actinomycin D (5 μ g/mL) was added to the cultures to stop new RNA synthesis at the time of MSA treatment, and hTERT mRNA levels were measured by quantitative reverse transcription-PCR (qRT-PCR) at the 2-, 4-, and 6-hour time points. The turnover of hTERT mRNA was determined by comparing mRNA levels over time in cells treated with or without MSA.

Quantitative nuclear run-on assay

Run-on transcription was done according to a previously described method with biotin-16-UTP (Roche; ref. 35). Biotin-labeled nascent transcripts were then isolated by using streptavidin particle beads and were quantitated by qRT-PCR.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out by using the ChIP-IT Express kit (Active Motif) per instruction of the manufacturer. Briefly, cells were treated with 1% formaldehyde for 15 minutes at room temperature. Nuclei were isolated, and chromatin was sheared to a size of 500 to 1,500 bp by sonication at 25% power for 10 pulses of 10 seconds each. Immunoprecipitation was done with an AR antibody (PG-21, Millipore) or rabbit IgG (as a negative control) overnight at 4°C. DNA released from the antibody-bound protein/DNA complexes was subjected to regular PCR and quantitative PCR analyses. The sequences of the primers used in regular PCR, –4 kb TERTs and –4 kb TERTas, were as described (8). The sequences of the primers and probes used in the quantitative PCR experiments were as

follows: *hTERT Primer-1*, 5'-TCACAGTGAAGAGGAA-CATGCCGT-3' (-3,844 to -3,820); *hTERT Primer-2*, 5'-TCAGTATCCCATGGAGGTGGCAGTTT-3' (-3,778 to -3,752); and *hTERT Probe*, 5'/56-FAM/AAGCCTG-CAGGCATCTCAAGGGAATT/3BHQ_1/-3' (-3,815 to -3,789).

Statistical analysis

Mean activities were calculated from at least three independent experiments done in triplicate. The Student's two-tailed *t* test was used to determine significant differences between two groups.

Results

Downregulation of *hTERT* by androgen-signaling suppression

We first performed dose-response and time course experiments on LNCaP cells cultured in a medium containing charcoal-stripped FBS to study the effect of androgen (in the form of DHT) on the expression of *hTERT*. Consistent with previous reports (7, 8), DHT treatment of LNCaP cells led to a marked induction of *hTERT* mRNA (Fig. 1A and B). The induction was dose dependent between 0.1 and 1 nmol/L DHT, and no further increase was observed when DHT was raised to 10 nmol/L (Fig. 1A). Based on this finding, we chose 1 nmol/L DHT for

the subsequent experiments. Figure 1B shows the data of the time course experiment. Expression of *hTERT* mRNA was increased by 2- or 4.5-fold at 16 or 24 hours, respectively. We also examined the effect of DHT on *hTERT* promoter activity by transiently transfecting LNCaP cells with the *hTERT*-3915/pGL4 construct, which contains nucleotides -3,915 to +40 of the *hTERT* promoter region. A significant increase of *hTERT* promoter activity was detected after treating the cells with DHT for 6 hours. The induction was almost completely blocked by 5 μ mol/L bicalutamide, which is an anti-androgen (Fig. 1C). We then studied the consequence of AR knockdown on *hTERT* expression. As shown in Fig. 1D, the knockdown of AR by treating LNCaP cells stably transfected with a doxycycline-inducible AR-shRNA lentiviral system with doxycycline led to a significant reduction of *hTERT* mRNA. The data therefore suggest that suppression of AR signaling by either an anti-androgen or AR knockdown could efficiently inhibit *hTERT* expression.

Downregulation of *hTERT*/telomerase by MSA

Because MSA reduces AR abundance (13, 14), it would be important to find out whether AR repression by MSA could also lead to *hTERT* downregulation. We treated LNCaP cells with various concentrations of MSA for 16 hours and determined *hTERT* mRNA levels by qRT-PCR. As shown in Fig. 2A, as little as 2.5 μ mol/L MSA

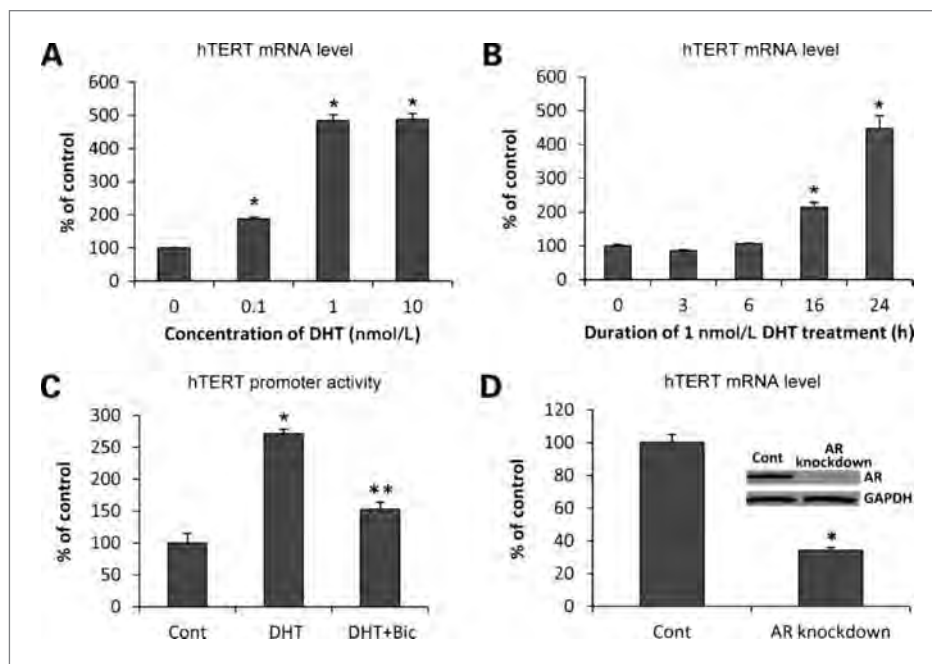


Figure 1. Androgen-AR regulation of *hTERT*. A and B, qRT-PCR analysis of *hTERT* mRNA level in LNCaP cells treated with different doses of DHT for 24 h (A) or 1 nmol/L DHT for 3, 6, 16, or 24 h (B). C, change in *hTERT* promoter activity, as determined by reporter gene assay, in LNCaP cells as a response to treatment with 1 nmol/L DHT or 1 nmol/L DHT + 5 μ mol/L bicalutamide (Bic) for 6 h. D, decrease of *hTERT* mRNA level, as assessed by qRT-PCR, in response to AR knockdown in LNCaP cells stably transfected with a doxycycline-inducible AR shRNA. Western blot confirmation of AR protein level is shown in the inset. Data are presented as percentage of ethanol-treated control cells cultured in charcoal-stripped FBS (A–C) or nontreated (no doxycycline) LNCaP cells stably transfected with a doxycycline-inducible AR shRNA (D). Bars, SEM. *, *P* < 0.01 compared with control. **, *P* < 0.01 compared with DHT-treated sample. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

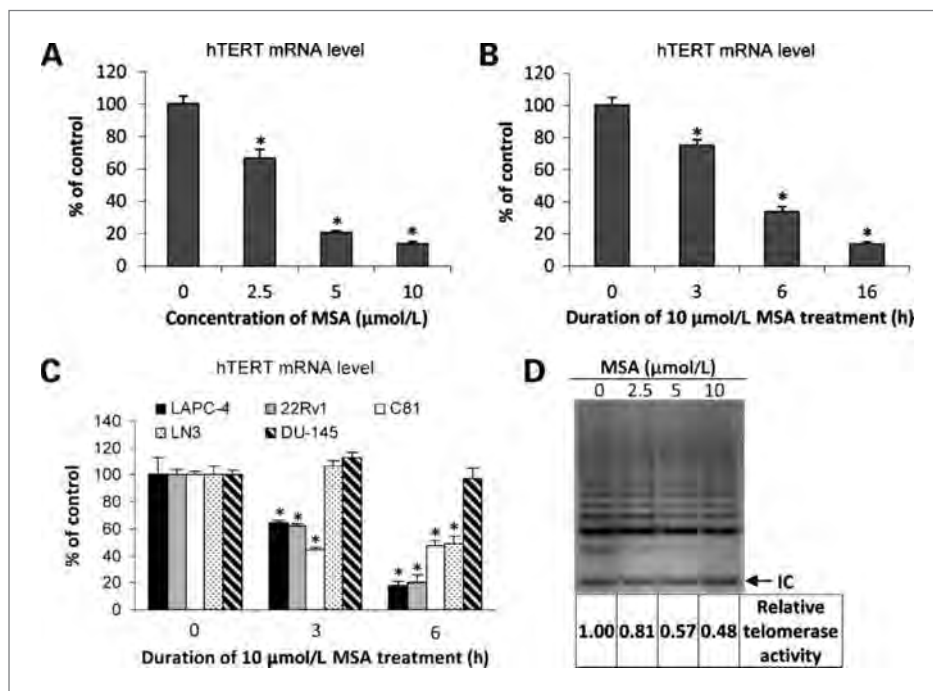


Figure 2. MSA suppression of *hTERT* expression and telomerase activity. **A**, changes in *hTERT* mRNA, as determined by qRT-PCR, as a function of MSA concentration at 16 h in LNCaP cells. **B** and **C**, expression of *hTERT* mRNA in response to treatment with 10 μmol/L MSA for various durations in LNCaP cells (**B**) or in LAPC-4, 22Rv1, C81, LN3, and DU-145 cells (**C**). Data are presented as percentage of nontreated control cells cultured in regular FBS. *, $P < 0.01$, significantly different from nontreated control. **D**, TRAP assay of changes in telomerase activity as a function of MSA concentration in LNCaP cells. IC, internal control. The numbers below the gel are normalized telomerase activities relative to nontreated control.

depressed *hTERT* mRNA level by 40%. Increasing the concentration of MSA to 5 or 10 μmol/L further reduced the level by 80% or more. The downregulation of *hTERT* mRNA was also time dependent (Fig. 2B). The level of *hTERT* mRNA declined gradually from 80% of control at 3 hours to 10% of control at 16 hours in response to 10 μmol/L MSA treatment. It is of note that the experiment was done on LNCaP cells cultured in a medium containing regular FBS and treated with MSA for various durations. As shown in Fig. 1B, a 16-hour treatment was required for androgen to induce *hTERT* mRNA level. The use of regular FBS allowed us to determine the time necessary for MSA to downregulate *hTERT* in a condition in which AR is already activated.

Similar decreases of *hTERT* mRNA were also observed in another androgen-dependent cell line, LAPC-4, as well as in three AR-positive castration-resistant cell lines, 22Rv1, C81, and LN3, but not in the AR-negative cell line DU-145 (Fig. 2C). The data shown in Fig. 2C were collected at 3 and 6 hours. We also analyzed *hTERT* mRNA level in DU-145 cells at 16 and 24 hours, and still could not detect any decrease by MSA (data not shown). Our results thus suggest a universal effect of MSA inhibition of *hTERT* in AR-positive prostate cancer cells. We wanted to examine the effect of MSA on *hTERT* protein level. However, none of the three *hTERT* antibodies we tested (Rockland Immunochemicals for Research, Santa Cruz Biotechnology, and GeneTex) produced a specific signal on Western blots, even with the use of nuclear extracts (see the Supplementary Data). This could be due to the relatively low level of *hTERT* protein expressed by the cells. To circumvent this problem, we analyzed telomerase activity in response to

MSA treatment in LNCaP cells. As shown in Fig. 2D, MSA treatment led to a dose-dependent inhibition of telomerase activity, although the magnitudes of inhibition are smaller than that observed on *hTERT* transcript level (refer to Fig. 2A for the transcript data). The disparity could be due to different sensitivity of the two assays and/or inherent difference between the extents of effect on *hTERT* mRNA versus *hTERT* protein or telomerase activity. Nonetheless, the data collectively showed a marked downregulation of *hTERT* and telomerase by MSA.

Inhibition of *hTERT* transcription by MSA

To determine whether the MSA downregulation of *hTERT* was due to increased mRNA degradation or decreased transcription, we performed the mRNA stability assay. Actinomycin D (5 μg/mL) was added to the LNCaP cultures to stop new RNA synthesis at the time of MSA treatment, and *hTERT* mRNA levels were measured by qRT-PCR during a 6-hour treatment duration. The decay of *hTERT* mRNA was compared in cells treated with or without MSA. Because actinomycin D could be cytotoxic, we monitored cell growth for up to 8 hours and did not observe cell death or significant growth inhibition during this period. Treatment with 10 μmol/L MSA actually increased the stability of *hTERT* mRNA (Fig. 3A). We then studied the effect of MSA on *hTERT* transcription by quantitative nuclear run-on assay. Biotin-labeled nascent transcripts obtained by run-on transcription were isolated by using streptavidin particle beads. To differentiate the effect of MSA on transcription initiation versus transcription elongation, qRT-PCR analysis was conducted with a primer probe set corresponding to either the 5'-end or

the 3'-end of *hTERT* mRNA. For both sets of primer probe, MSA treatment resulted in approximately 50% and 80% inhibition of *hTERT* transcription at 3 and 6 hours, respectively (Fig. 3B). The data thus indicate that the MSA-mediated decrease of *hTERT* mRNA level is accounted for largely by a block of *hTERT* transcription initiation. Gene transcription is generally controlled by promoter regions. We next characterized the effect of MSA on the activity of the ~4-kb *hTERT* promoter in LNCaP cells cultured in a medium containing regular FBS. Treatment with 10 $\mu\text{mol/L}$ MSA caused a time-dependent inhibition of the promoter activity (Fig. 3C). When we repeated the experiment by culturing the cells in charcoal-stripped FBS and adding DHT to the culture, we also found that MSA was able to block DHT induction of *hTERT* promoter activity (Fig. 3D).

Reduction of AR occupancy of the *hTERT* promoter by MSA

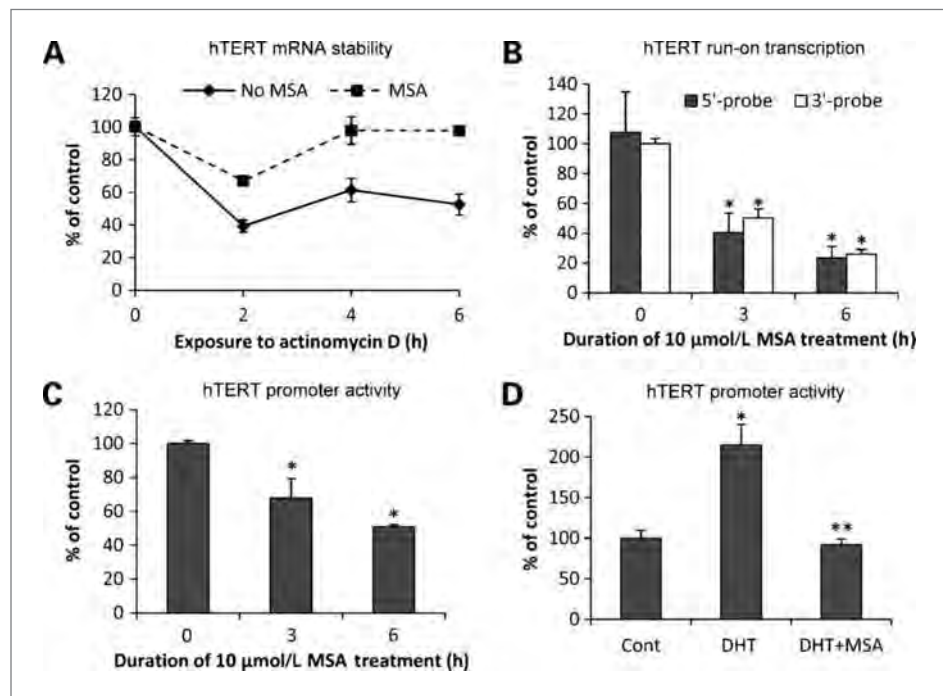
To show that suppressed *hTERT* promoter activity is attributable to reduced AR occupancy of the *hTERT* promoter as a consequence of MSA downregulation of AR, we performed the ChIP assay on the *hTERT* promoter region that was reported to associate with AR (8). Figure 4A shows the result of PCR amplification of DNA coimmunoprecipitated with either an AR antibody or IgG using primers specific for the *hTERT* promoter region. The specificity of the ChIP assay is evident from the difference in band intensity between the AR antibody-immunoprecipitated samples and the IgG-immunoprecipitated samples. In concordance with a previous report (8), AR is only weakly associated with the *hTERT*

promoter in the absence of androgen in LNCaP cells. DHT significantly increased the AR occupancy of the promoter, and the increase was blocked by 10 $\mu\text{mol/L}$ MSA. We then conducted quantitative PCR of the coimmunoprecipitated DNA to quantify the change in AR occupancy of *hTERT* promoter in response to different treatments. As shown in Fig. 4B, DHT treatment induced a ~6-fold increase of AR occupancy of the promoter, and the increase was completely suppressed by MSA.

Combinatorial effects of bicalutamide and MSA on *PSA* and *hTERT*/telomerase

Bicalutamide and MSA target AR through two distinct mechanisms. As an anti-androgen, bicalutamide suppresses AR *trans*-activation by competing with androgens for binding to AR. MSA also disrupts AR signaling mainly through reducing the availability of AR (13, 14). Therefore, it is reasonable to believe that the two drugs in combination would produce a more pronounced effect on AR signaling suppression. We quantified the mRNA level of *PSA*, a well-characterized AR target, by qRT-PCR in LNCaP and LN3 cell models. As shown in Fig. 5A, incubation of LNCaP cells with DHT for 6 hours led to a 3-fold increase of *PSA* mRNA compared with the vehicle control. The level was reduced to approximately 1.4- or 1.9-fold of the vehicle control as a result of treatment with 5 $\mu\text{mol/L}$ bicalutamide or 2.5 $\mu\text{mol/L}$ MSA, respectively. Combining bicalutamide and MSA at these concentrations brought down DHT-induced *PSA* expression to ~70% of the vehicle control. We chose to use low doses of bicalutamide or MSA individually to leave room for an enhanced effect with the

Figure 3. MSA inhibition of *hTERT* transcription. A, effect of MSA on *hTERT* mRNA stability. B, effect of MSA on *hTERT* transcription initiation as assessed by quantitative nuclear run-on analysis. C, effect of MSA on *hTERT* promoter activity. D, effect of MSA on DHT-induced *hTERT* promoter activity. The results are expressed as percentage of nontreated control cells cultured in regular FBS (A–C) or ethanol-treated control cells cultured in charcoal-stripped FBS (D). Bars, SEM. *, $P < 0.01$, significantly different from nontreated control. **, $P < 0.01$, significantly different from DHT-treated sample.



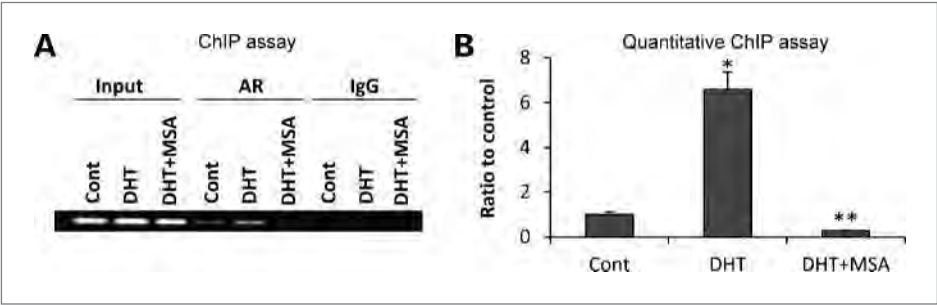


Figure 4. MSA reduction of AR occupancy of *hTERT* promoter as determined by ChIP assay (A) or quantitative ChIP assay (B). The assay was conducted at 16 h after treatment with 1 nmol/L DHT and/or 10 μ mol/L MSA. Input, total input chromatin for each treatment sample. AR, DNA coimmunoprecipitated with an AR antibody. IgG, DNA coimmunoprecipitated with an IgG. *, $P < 0.01$, significantly different from ethanol-treated control cells cultured in charcoal-stripped FBS. **, $P < 0.01$, significantly different from DHT-treated sample.

combination. Consistent with the castration-resistant characteristics of LN3 cells, only a marginal induction of *PSA* mRNA by DHT was observed (Fig. 5A). Treating LN3 cells with 10 μ mol/L bicalutamide or 5 μ mol/L MSA reduced DHT-induced *PSA* level to approximately 60% and 50% of the vehicle control, respectively. The combination, on the other hand, almost completely blocked the expression of *PSA* mRNA. The data thus show a robust suppression of AR *trans*-activation by bicalutamide and MSA in androgen-dependent and CRPC cells.

We proceeded to evaluate the effect of bicalutamide in combination with MSA on *hTERT* expression and telomerase activity. The *hTERT* mRNA data (Fig. 5B) essentially mirror the *PSA* mRNA result. In LNCaP and LN3 cells, treatment with bicalutamide or MSA alone inhib-

ited DHT-induced *hTERT* expression; the inhibitory effect was much more striking when the drugs were used in combination. We then analyzed telomerase activity as a result of the combination treatment in the two cell lines. As shown in Fig. 5C, telomerase activity in LNCaP cells was markedly induced by DHT. Bicalutamide or MSA alone produced minimal inhibition of DHT-induced telomerase activity. The two drugs in combination, however, almost completely blocked the DHT induction of telomerase activity. Comparing to the data presented in Fig. 2D, the effect of MSA alone seems to be smaller here (0% versus 19% inhibition). This could be due to the different culturing conditions that were used for the two experiments (charcoal-stripped FBS for Fig. 5C versus regular FBS for Fig. 2D). The combination effect

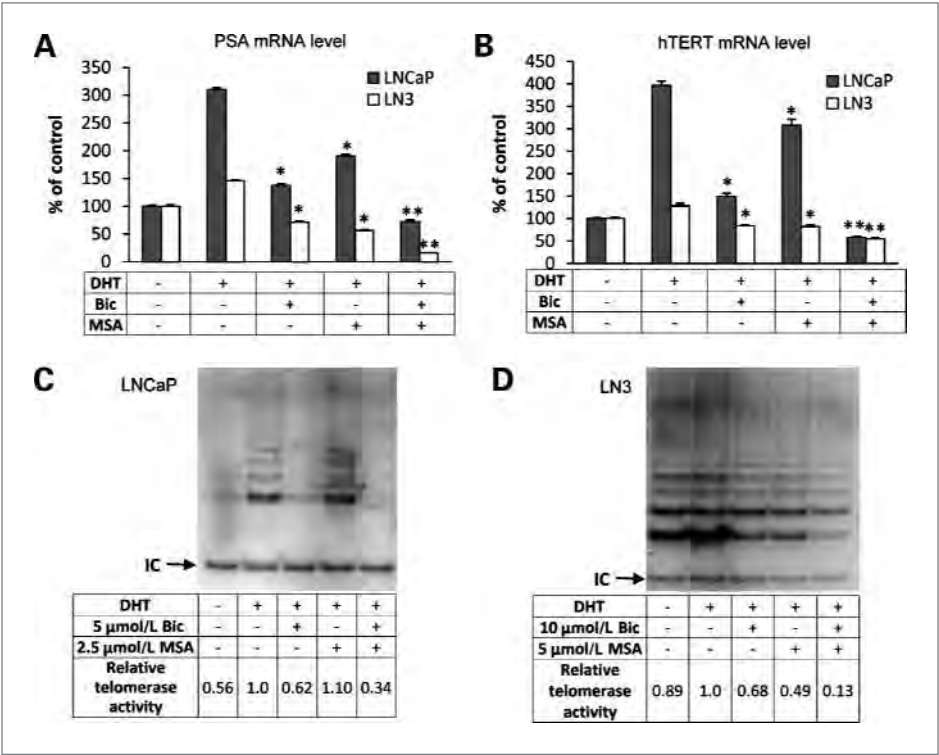


Figure 5. Bicalutamide (Bic) and MSA downregulation of *PSA* and *hTERT*/telomerase. LNCaP cells were treated with 5 μ mol/L Bic and/or 2.5 μ mol/L MSA, and LN3 cells with 10 μ mol/L Bic and/or 5 μ mol/L MSA. The levels of *PSA* mRNA (A) and *hTERT* mRNA (B) were determined by qRT-PCR. The activity of telomerase in LNCaP (C) and LN3 (D) cells were analyzed by the TRAP assay. Data in A and B are expressed as percentage of ethanol-treated control cells cultured in charcoal-stripped FBS. Bars, SEM. *, $P < 0.01$ compared with DHT-treated sample. **, $P < 0.01$ compared with Bic- or MSA-treated sample. IC (C and D), internal control. The numbers in C and D are normalized telomerase activities relative to the DHT-treated sample.

on telomerase activity was also manifested in LN3 cells (Fig. 5D). Thus, the telomerase activity data were qualitatively similar to the *hTERT* mRNA result.

Combinatorial effects of bicalutamide and MSA on apoptosis

Given the critical role of hTERT/telomerase in regulating cell survival, we assessed the effect of bicalutamide and MSA on apoptosis induction in the absence or presence of hTERT overexpression. LNCaP and LN3 cells were treated with bicalutamide and/or MSA for 48 hours, and apoptosis was analyzed by using the Cell Death ELISA assay. As shown in Fig. 6A and B, in both cell models, bicalutamide and MSA in combination led to a more striking increase of apoptosis than either agent alone. Conversely, the combination efficacy was not observed in the AR-negative DU-145 cell line (Fig. 6C). In concordance with the previously reported AR-independent activity of MSA in prostate cancer cells (31, 36–39), MSA treatment of DU-145 cells resulted in a marked induction of apoptosis. However, the combination with bicalutamide was not able to lead to further induction of apoptosis.

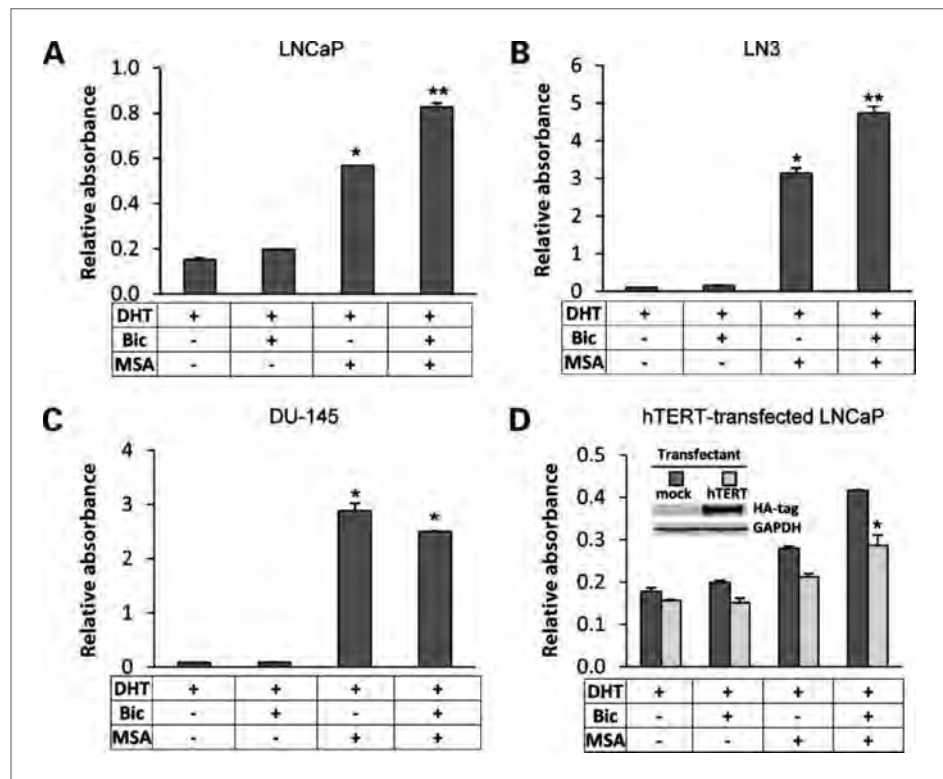
To delineate the functional significance of *hTERT* down-regulation in mediating the effect of bicalutamide and MSA, we transiently transfected LNCaP cells with an hTERT expression construct and assessed the response of the hTERT-overexpressing cells to the induction of apoptosis. The apoptosis assay was conducted at 24 hours after treatments, and the data are presented in Fig. 6D. An abundant presence of hTERT not only weakened the

apoptosis-inducing ability of bicalutamide or MSA alone but also significantly inhibited the apoptosis-inducing efficacy of the combination treatment. One reason that the difference was seemingly compressed was due to the fact that only a fraction of cells was successfully transfected, and apoptosis was assessed using the whole cell population. It is of importance to point out that, to obtain uniform transfection efficiency, the transfection was carried out in a 10-cm culture dish, and the cells were subsequently trypsinized and replated onto a 96-well plate for apoptosis analysis. Because the cells were allowed to recover for an additional 48 hours before treatment, and transiently transfected plasmid generally could not maintain expression beyond 72 hours after transfection, the apoptosis assay in this experiment was conducted at 24 hours after treatments, not at 48 hours as in Fig. 6A. This could contribute to the difference in the magnitude of apoptosis induction observed in the experiments presented in Fig. 6A and D.

Discussion

ADT is the mainstay treatment for advanced prostate cancer (40). However, relapse with CRPC invariably occurs. Because CRPC is lethal and incurable (40), developing effective strategies to enhance the efficacy of ADT and circumvent resistance becomes an urgent task. AR expression and signaling are maintained in CRPC although the tumor is no longer responsive to ADT. Prostate cancer can adapt to androgen deprivation by AR

Figure 6. Bicalutamide (Bic) and MSA induction of apoptosis. A to C, induction of apoptosis as determined by the Cell Death ELISA assay in LNCaP cells treated with 5 $\mu\text{mol/L}$ Bic, 2.5 $\mu\text{mol/L}$ MSA, or Bic and MSA in combination (A); in LN3 cells treated with 10 $\mu\text{mol/L}$ Bic, 5 $\mu\text{mol/L}$ MSA, or Bic and MSA in combination (B); or in DU-145 cells treated with 10 $\mu\text{mol/L}$ Bic, 2.5 $\mu\text{mol/L}$ MSA, or Bic and MSA in combination (C) for 48 h. The data obtained by ELISA were normalized by SRB results for cell content. *, $P < 0.01$ compared with DHT-treated sample. **, $P < 0.01$ compared with Bic- or MSA-treated sample. D, attenuation of Bic/MSA induction of apoptosis by ectopic expression of hTERT in LNCaP cells. The apoptosis assay was conducted at 24 h after Bic/MSA treatments. Inset, Western blot confirmation of the level of transfected HA-tagged hTERT. *, $P < 0.01$ compared with mock transfectants.



mutation, AR overexpression, androgen-independent activation of AR, and/or by increasing intratumoral androgens through *de novo* steroidogenesis (41–43). Xenograft studies have shown that knocking down AR expression by shRNA could delay the progression of prostate cancer to CRPC and suppress the growth of prostate tumor that has already progressed to the castration-resistant state (32, 44). Therefore, rationally designed therapies aimed at diminishing the availability of AR would be helpful not only in enhancing the efficacy of ADT, but also in inhibiting the development of CRPC. In this study, we reported for the first time that MSA, an agent that could effectively reduce AR abundance, has the ability to potentiate the cancer killing efficacy of bicalutamide in androgen-dependent and CRPC cells.

Because bicalutamide and MSA suppress AR signaling at different points, a combination of the two drugs would be expected to deliver a more powerful punch in knocking out AR signal transduction. In support of this notion, our data showed that the combination produced a stronger suppression of PSA and *hTERT* than either agent alone. We also showed that apoptosis induction by the two agents was mitigated by the restoration of *hTERT*, thus confirming the critical involvement of *hTERT* downregulation in mediating the combination effect. The downregulation of *hTERT* occurred mainly at the transcriptional level, and decreased AR occupancy of the *hTERT* promoter contributes to the reduction of *hTERT* transcription. These findings, however, cannot exclude the possibility of the existence of an AR-independent mechanism by which MSA diminishes *hTERT* expression. This possibility deserves further investigation.

The role of telomerase is generally regarded as maintaining telomere length. However, accumulating evidence has indicated that telomerase may have additional functions in regulating cell growth and survival, and thereby oncogenesis (45). Telomeric DNA repeats are associated with an array of telomere-specific proteins in forming a chromosome-protective cap (45). Functional telomeres switch stochastically between capped and uncapped states (46). Telomerase plays an essential role in telomere recapping. Uncapped telomeres, if left uncorrected too long, are recognized as DNA breaks and will trigger a DNA damage response, leading to cell cycle arrest and/or apoptosis (46). Additionally, the nonhomologous end joining of dysfunctional telomeres may result in telomere-telomere fusions and contribute to genomic instability. In the present study, we show that bicalutamide and MSA induce a marked increase of apoptosis at 48 hours. Telomerase suppression by bicalutamide and

MSA is unlikely to result in appreciable telomere shortening within such a short period of time. This is actually confirmed by our experiment (data not shown). Therefore, a mechanism independent of telomere shortening should be considered. It is likely that inhibiting telomerase by bicalutamide and MSA could lead to telomere uncapping, which would then trigger a rapid DNA damage response and lead to cell cycle arrest and apoptosis. Experiments are ongoing to test the hypothesis.

The identification of *hTERT*/telomerase as an important AR target mediating the bicalutamide/MSA effect has great clinical implications. Telomerase activation has been reported in >90% of prostate cancer samples, but not in normal or benign prostatic hyperplasia tissues (47). Telomerase activation has been well documented to play an essential role in cell survival and oncogenesis, and inhibition of telomerase has been shown to suppress growth and tumorigenic potential of prostate cancer cells (3, 48). Blocking telomerase activation by anti-androgen and MSA through suppressing AR signaling could thus represent an effective and selective treatment modality to target prostate cancer cells. In addition, *hTERT*/telomerase could be measured in blood and urine (49, 50) and, therefore, could serve as a noninvasive, tumor-specific, functionally relevant molecular biomarker for monitoring the efficacy of the intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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