

AD _____

Award Number: W81XWH-04-1-0839

TITLE: Restoration of Transforming Growth Factor beta Signaling by Histone Deacetylase Inhibitors in Human Prostate Carcinoma□□

PRINCIPAL INVESTIGATOR: Zheng D. Qian, Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21231

REPORT DATE: October 2006

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01/10/06		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 15 Sep 2004 – 14 Sep 2006	
4. TITLE AND SUBTITLE Restoration of TGFb signaling in human prostate cancer by histone deacetylase inhibitor MS-275				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0839	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Zheng D. Qian, Ph.D. E-Mail: zqian1@jhmi.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University Baltimore, MD 21231				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: The goal of the current grant is to investigate the potential antitumor activity of histone deacetylase inhibitor MS-275 along with the activation of TGFb signaling pathway with the restoration of TGFb receptor II. As presented in our initial proposal, prostate cancer cell line LNCaP has reduced expression in TGFbRII, which is due to the promoter histone deacetylation. Subsequent treatment with chromatin remodeling agent MS-275 was able to restore the expression of TGFbRII. We hypothesized that the restoration of TGFb signaling may contribute to the antitumor activity of MS-275. In the past a year and half, we have focused our effort to identify the re-expression of TGFbRII in vivo, and investigate the antitumor activity of MS- 275 in several relevant prostate cancer model. We observed dose and time dependent upregulation of TGFb1 and TGFbRII in LNCaP cells. A cDNA microarray of LNCaP cells (untreated vs. MS-275 treated) has been done, and the data is being analyzed. We observed the antitumor activity of MS-275 in two different models of prostate cancer xenografts. Immunohistochemistry analysis of reactivation of TGFb1 in vivo is on going.					
15. SUBJECT TERMS prostate cancer, Histone deacetylase inhibitors, TGFb signaling					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....4.

Body..... 4-5.

Key Research Accomplishments..... 5.

Reportable Outcomes.....5.

Conclusions.....5.

References.....

Appendices.....

SUPPORTING DOCUMENTS6-11

Final Report:

Introduction:

The goal of the current grant is to investigate the potential antitumor activity of histone deacetylase inhibitor MS-275 along with the activation of TGF β signaling pathway with the restoration of TGF β receptor II. As presented in our initial proposal, prostate cancer cell line LNCaP has reduced expression in TGF β RII, which is due to the promoter histone deacetylation. Subsequent treatment with chromatin remodeling agent MS-275 was able to restore the expression of TGF β RII. We hypothesized that the restoration of TGF β signaling may contribute to the antitumor activity of MS-275. In the first one and half year of study, we had focused our effort to identify the re-expression of TGF β RII in vivo, and investigate the antitumor activity of MS-275 in several relevant prostate cancer model. Since the last progress report, we have focused our effort to demonstrate a) the epigenetic mechanism of TGF β RII expression by HDAC inhibitors, b) the effect of HDAC inhibitors on androgen receptor signaling.

Body:

Specific Aim 1): To determine whether loss of TGF β RII expression in a human prostate carcinoma cell line is due to transcriptional epigenetic repression.

To demonstrate the loss of expression of TGF β RII in LNCaP cells, we performed chromatin immunoprecipitation assay (CHIP). As shown in Figure 1C, the promoter region of TGF β RII and its primary ligand TGF β 1 were un-acetylated in control (drug free) condition. Addition of MS-275 at 1 and 2 uM increased the promoter acetylation, which corresponds to the upregulation of the gene expression (as demonstrated in figure 1A 1B). The current CHIP assay (figure 1C) complements with the previous RT-PCR results (figure 1a 1b), establish the epigenetic silencing nature of these two genes in prostate cancer LNCaP cells.

Specific Aim 2): To determine whether re-expression of TGF β RII by HDACI is associated with the restoration of TGF β signaling and contributes to HDACI antitumor activity.

We also examined the TGF β RII expression levels in other prostate cancer cell lines – PC3, LAPC4, and DU145. All these cell lines exhibited robust expression level of TGF β RII and its ligands. HDAC inhibitors could not further upregulate the expression. In addition, the Smad pathway components that downstream of TGF β RII signaling in all these cell lines were not altered by the HDAC inhibition. Therefore, we concluded that the upregulation of TGF β RII signaling by HDAC inhibitors were limited to the LNCaP cells. In vitro, cell proliferation assays confirmed statistically significant inhibition over single agents when HDAC inhibitors were combined with exogenous TGF β 1 (figure 2). However, due to the fact that HDAC inhibitors also target many growth pathways that are independent of TGF β RII signaling, there were significant levels of inhibition on the growth of cancer cells both in vitro and in vivo regardless of the TGF β RII status (table 1, figure 3, 4 and 6). The conclusion, therefore, is that HDAC inhibitors may upregulate TGF β RII that partially mediates the antitumor activity of HDAC inhibitors.

Specific Aim 3): To evaluate the antitumor effect of HDACIs in combination with anti-androgen treatment (androgen ablation).

Our initial hypothesis is that HDAC inhibitors may synergies with androgen ablation therapy. However during our in vitro experiments and clinical trials of HDAC inhibitors in prostate cancer patients, we observed that HDAC inhibitor's antitumor activity does not complement with the reduction of androgen ablation. In vitro, HDAC inhibitors may upregulate PSA expression, which is a target gene of androgen signaling pathway under total growth factor and androgen starvation condition (table 2), but not in full serum condition (figure 5). The possible modulation of PSA by HDAC inhibitors has not been fully investigated. Under our experimental conditions, we did not observed PSA induction in hormone sensitive cell lines at the gene expression level, but

we observed increased protein levels in LAPC4 treated cells. These results suggest a possible PSA modulation by MS-275 at the posttranscriptional level, or perhaps by increasing its secretion. Further studies will be necessary to elucidate this observation. The induction of PSA protein level in association with inhibition of cell proliferation following in vitro MS-275 treatment suggest that changes in PSA levels should not be considered a primary endpoint in clinical trials involving HDAC inhibitors. However, PSA induction may represent a readable biological effect of HDAC inhibition and a potential biomarker for drug exposure.

Key Research Accomplishment

1. MS-275 upregulated both TGFb1 and TGFbRII expression in LNCaP cells
2. MS-275 was effective in vivo targeting LNCaP growth
3. The growth inhibition of MS-275 was cytostatic with G1 arrest.
4. MS-275 was also effective against prostate cancer cell growth in the bone environment
5. Established the epigenetic nature of TGFBR2 and TGFb1 silencing in LNCaP cells.
6. Observed the PSA induction by MS-275 that is in contrast with the antitumor activity of the drug.

Reportable Outcome

A manuscript reporting the in vivo antitumor activity of MS-275 in various pre-clinical prostate cancer models has been submitted it to the journal of prostate.

Conclusion

MS-275 was effective against prostate cancer growth in vitro and in vivo. The re-expression of TGFbRII and its ligand TGFb1 in LNCaP cells may be mediators for the MS-275 antitumor activity. However, the upregulation of TGFb1 may be able to promote the tumor microenvironment facilitating tumor growth.

Although a clinical role of HDAC inhibitors is already a reality, their general clinical utility will likely depend greatly on the future development of molecular or cellular predictors of their antitumor activity. Molecular targeted combination therapies of post-transcription modifiers such HDAC inhibitors with standard therapies (chemotherapy and radiation therapy) and novel biologics including angiogenesis inhibitor represent an exciting challenge. Understanding the nature of the molecular basis of the selectivity of HDACs and designing specific HDACs inhibitors will be critical to further exploit the potential of this novel class of agents in the treatment of solid tumors including prostate cancer.

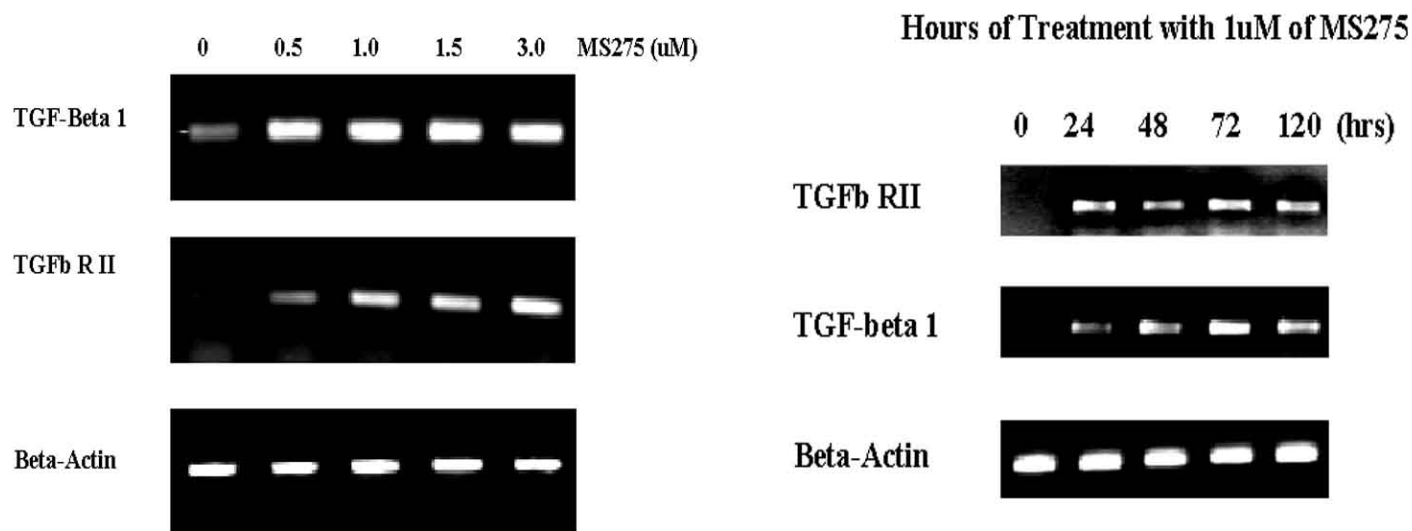


Figure 1 A. Dose-dependent gene upregulation of TGFb1 and TGFbRII in LNCaP cells after MS-275 treatment.

Figure 1B. Time-dependent gene upregulation of TGFb1 and TGFbRII in LNCaP cells after MS-275 treatment.

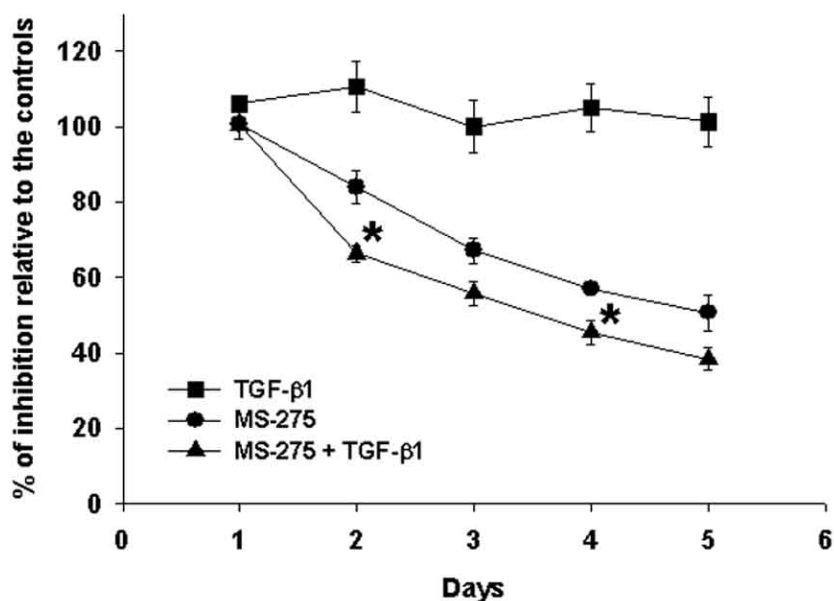


Figure 2. Cell proliferation assay of LNCaP with solvent control, MS-275, TGFb1 only, and as combination. * P<0.05 vs. single agent student t test.

Chromatin Immunoprecipitation PCR (CHIP)

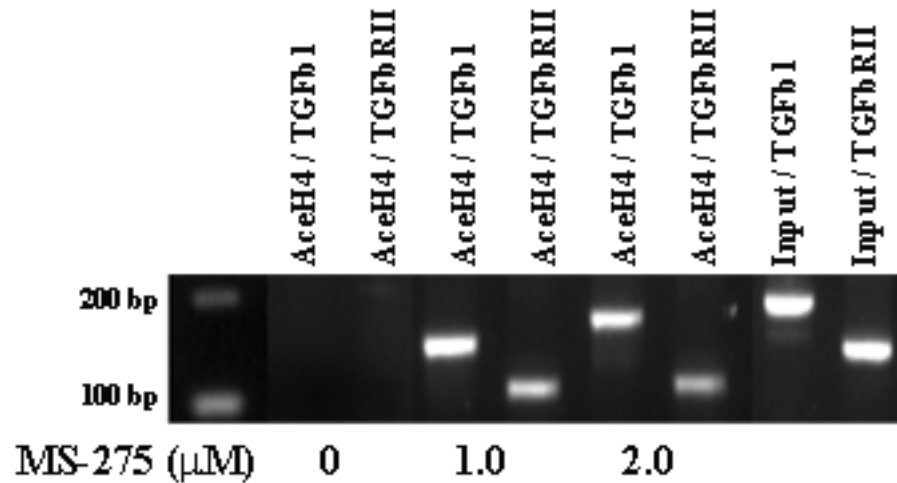


Figure 1C.

LNCaP cells were treated with 1 or 2 μ M of MS-275 for 24 hours, cells were subject to CHIP assay using Acetyl-H4 antibody (Upstate) based on the protocol included in the CHIP assay kit (Upstate), and DNA fragments containing acetyl-H4 were amplified using PCR primers specific for TGFb1 and TGFbRII promoters. Total DNA without acetyl-H4 immunoprecipitation was used as positive input control.

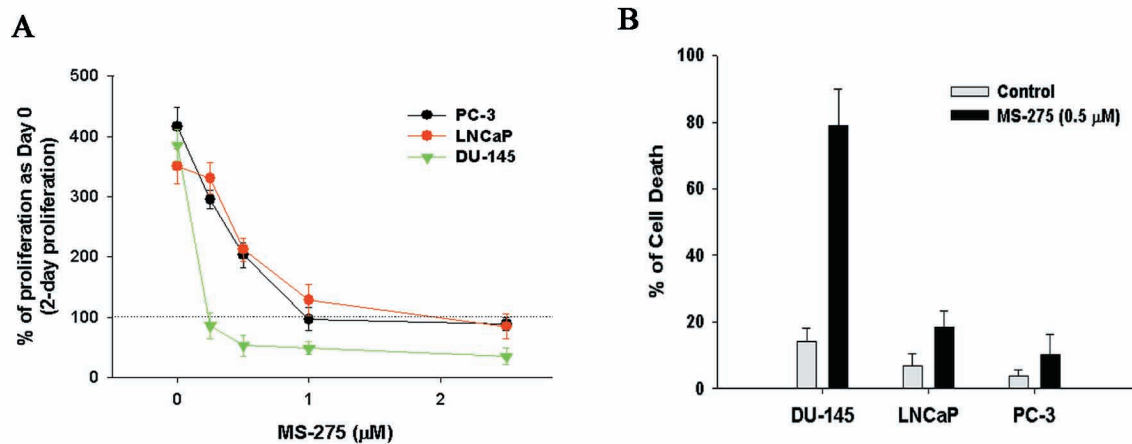


Figure 3. A) PC-3, DU-145 and LNCaP cells were treated with 0, 0.1, 0.5 1.0 and 2.0 μM of MS-275 for 48 hours. The viable cell numbers at Day 2 were measured, and the viable cells numbers at day 0 were normalized to 100%. B) Similar experiments were done as in A) using 0.5 μM of MS-275. Then cells were harvested and stained with Annexin V and PI, and analyzed with FACS.

Table 1. Cell cycle analysis of three prostate carcinoma cell lines with and without MS-275 treatment (0.5 μM for 48 hours). The cell population at each specific stage was expressed as % (control -> treated).

	SubG0 (%)	G0/G1 (%)	S (%)	G2/M (%)
DU-145	5 -> 75	55 -> 13	19 -> 9	21 -> 3
LNCaP	1 -> 4	62 -> 73	17 -> 5	20 -> 22
PC-3	7 -> 10	44 -> 52	24 -> 10	25 -> 28

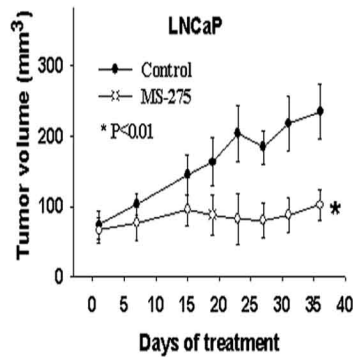


Figure 4. Approximately 2 million LNCaP cells were injected into male nude mice s.c. When the tumors were palpable, daily MS-275 at 20 mg/kg/day were administrated for 5 weeks. Tumor volumes were followed with caliper measurements. Data were mean and SEM, * $P < 0.05$, student t test.

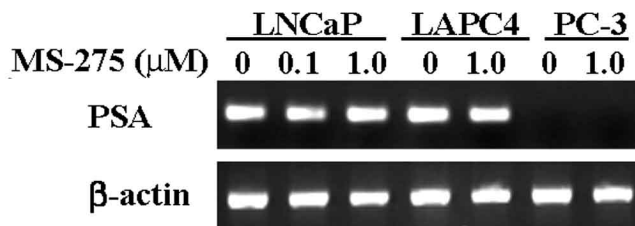


Figure 5. PSA gene expression in LNCaP and LAPC4 cells were analyzed by RT-PCR with and without MS-275. PC-3 cell line that does not express PSA was used as a negative control.

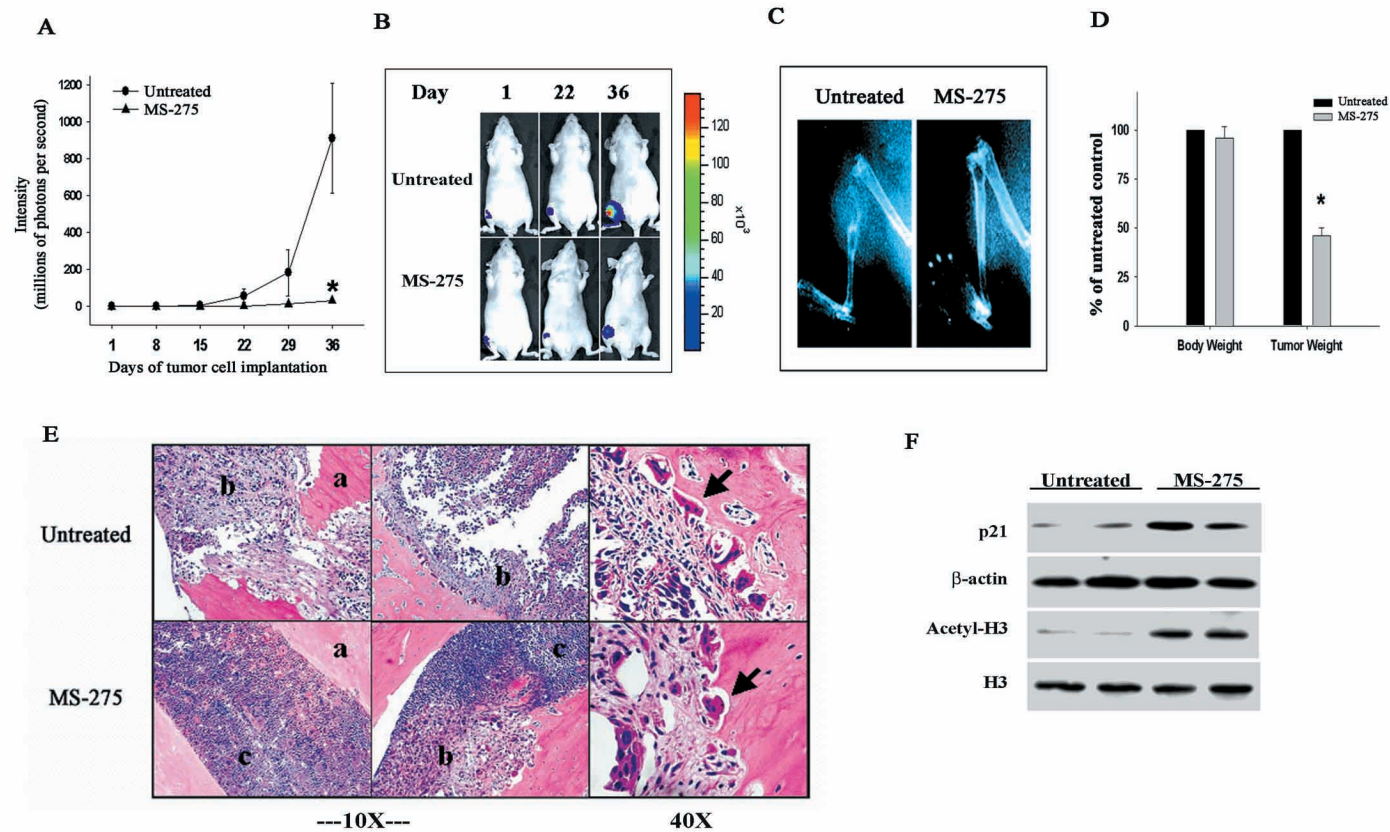


Figure 6. (A) Tumor burden in the control and MS-275 treated groups were followed by *in vivo* imaging of luciferase activity. The values for each group was expressed as mean and standard error, *, $P < 0.05$. (B) Representative luciferase imaging of one mouse from each group at day 1, 22 and 36 post-injection. (C) Representative radiography of tumor bearing legs in control and MS-275 treated mice at the end of the treatment. (D) The body weight and tumor weight of the mice at the end of the experiment expressed as % of the untreated control group with standard deviation. The net tumor weights were obtained by subtracting the normal contralateral tibia from the tumor-bearing tibia. *, $P < 0.05$. (E) Representative H&E at 10X and 40X magnification. a = bone matrix, b = tumor cells, c = bone marrow cells. Osteoclasts were shown as multinuclear cells indicated by arrow. (F) Western blots of tumor tissue samples showing increased histone H3 acetylation, and p21 expression in MS-275 treated mice.

Table 2.

Cell Line	Treatment	Total PSA (ng/ml)	Total Protein (mg/ml)	Normalized PSA (ng/mg)	FOLD CHANGE over control
LAPC4 p46	Control	1.84	4.34	0.42	1.00
LAPC4 p46	1 μ M MS-275	4.02	4.25	0.95	2.23
LAPC4 p77	Control	3.15	4.32	0.73	1.00
LAPC4 p77	1 μ M MS-275	7.95	3.88	2.06	2.82
PC3	Control	<0.1	5.05	ND	ND
PC3	1 μ M MS-275	<0.1	4.81	ND	ND

PSA production was examined in early and late passages of the prostate cancer cell lines LAPC4 and PC3. Androgen-dependent cell line LAPC4 demonstrated two-fold induction of PSA after 24hr treatment with 1 μ M MS-275. No PSA production was observed in androgen receptor-negative prostate cancer cell line PC3.