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14. ABSTRACT Prostate cancer (PCa) is the most common invasive malignancy and second leading cause of cancer death in men in the United States. Up till now, hormone ablation therapy is the major way to treat PCa. Such therapy only causes a temporary regression and tumor growth resumes within 6-18 months. Therefore, better androgen blockade is not the answer for treating PCa. Rather, research efforts should focus on the therapeutic agents that will inhibit growth factor signaling pathways thereby inhibit growth. A large number of studies have pointed out that inositol hexaphosphate (IP6) could have beneficial effect on variety of cancers. The specific aims of this proposal are to determine (1) the in vivo effects of IP6 on the growth of PCa (2) the efficacy of IP6 in inhibiting growth factor-induced DNA synthesis of the PCa cells in vitro, and (3) the molecular mechanisms by which IP6 inhibits growth of PCa cells. The information we obtain from these experiments will provide a better understanding of the potential role of IP6 in the prevention of growth of PCa cells. This information will lead to more effective PCa prevention and treatment strategies in human that might prolong the longevity of men with prostate cancer.					
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## **INTRODUCTION:**

Prostate cancer is the most common invasive malignancy and second leading cause of cancer death in men in the United States and many other parts of the world. Up till now, hormone ablation therapy is the major way to treat prostate cancer. Such therapy only causes a temporary regression and tumor growth resumes within 6-18 months. It is now well established that aberrant expressions of mitogenic growth factors and their receptors are responsible for unregulated growth of the prostate cancer. Once autocrine growth factor loops are operative, prostate cancer progresses to an androgen-independent state. It is uniformly fatal because no systemic therapy currently exists that inhibit growth of androgen-independent prostate cancer. Therefore better androgen blockade is not the answer for treating prostate cancer. Rather, research efforts should focus on the therapeutic agents that will inhibit growth factor signaling pathways thereby inhibit growth. While many new classes of cancer chemopreventive agents are being evaluated in clinical trials for other malignancies, little success has been achieved in terms of prostate cancer prevention. During the past several years, a large number of studies have pointed out that inositol hexaphosphate (IP6), the most abundant phosphorylated inositol present in beans, cereal grains, lentils and legumes, could have beneficial effect on variety of cancers. The underlying hypothesis driving our work is that unregulated expression of mitogenic growth factors are responsible for carcinogenesis of the prostate gland and IP6 can prevent such development by inhibiting growth factor-induced signal transduction. Therefore, IP6 could be a potential agent for the prevention and treatment of prostate cancer. The specific aims of this project were to examine (1) the in vivo effects of IP6 on the growth of prostate cancer (2) the efficacy of IP6 in inhibiting growth factor-induced DNA synthesis of prostate cancer cells in vitro, and (3) to determine the molecular mechanisms by which IP6 inhibits growth of prostate cancer cells.

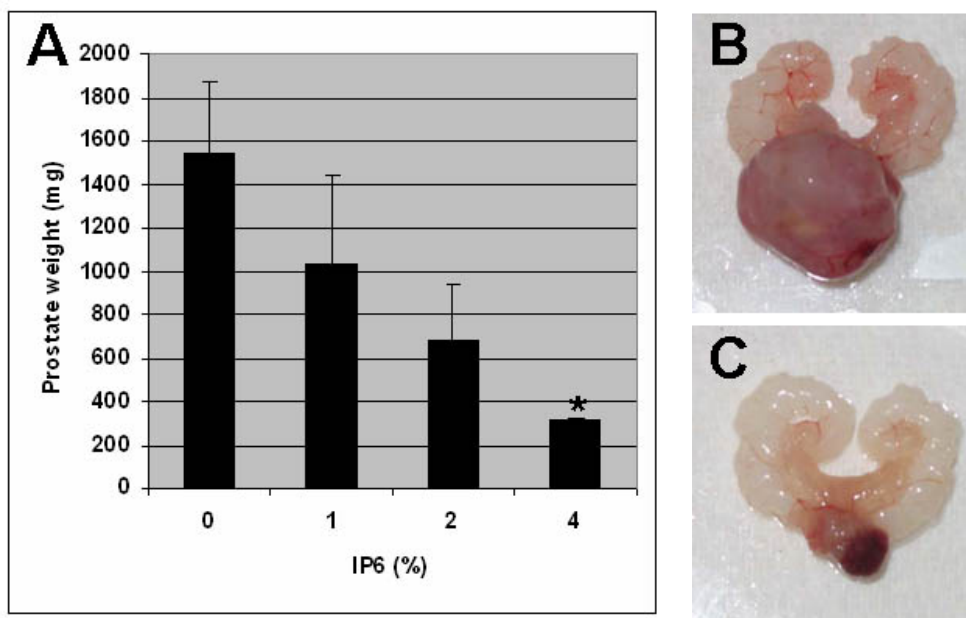
## **BODY:**

In my proposal under the “Statement of Work”, I proposed that my first task would be to determine the in vivo effects of inositol hexaphosphate (IP6) on the growth and development of prostate cancer in TRAMP mice. To test the efficacy of IP6 in preventing prostate cancer growth, 32 male TRAMP mice of 4 weeks of age were treated with 1, 2 and 4% IP6 or without IP6. As seen in Figure 1A, IP6 dose-dependently decreased prostate tumor growth over 32 weeks of treatment. Although, we observed a dose-dependent decrease in tumor growth, significant inhibition was only observed in 4% IP6-treated groups. In control diet groups, tumor was very large in size and was exclusively in the prostate gland whereas seminal vesicle was normal (Fig. 1B). IP6 (4%) treatment inhibited such tumor growth in the prostate (Fig 1C). We submitted one manuscript on mechanism of IP6 action of telomerase regulation and preparing another two manuscripts.

## **KEY RESEARCH ACCOMPLISHMENTS:**

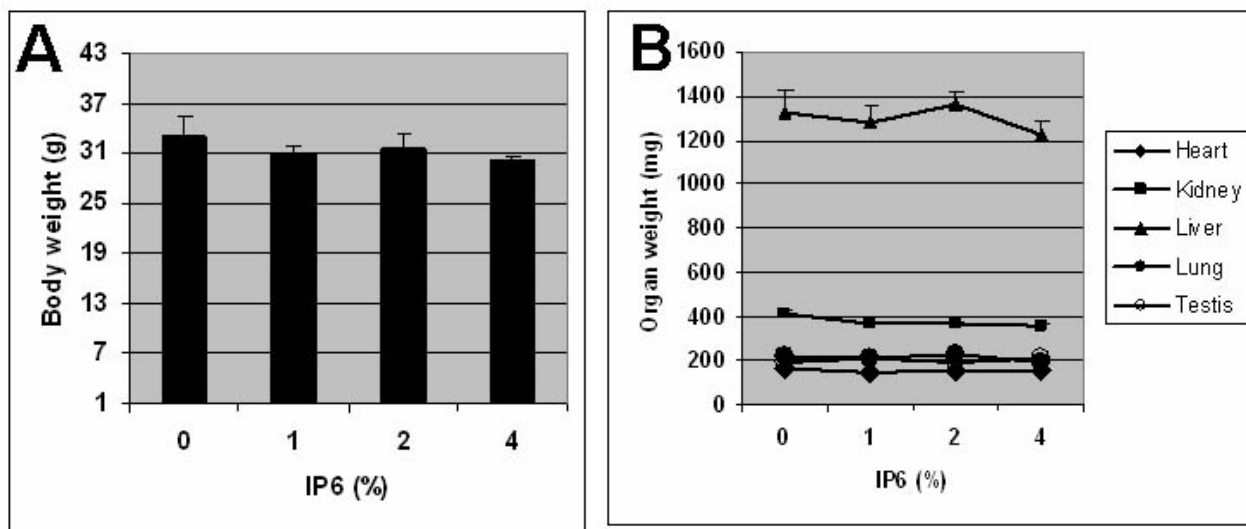
- In vivo treatment of IP6 to TRAMP mice is complete (Task 1)
- Mechanism of growth inhibition by IP6 has been resolved (Task3)
- Efficacy of IP6 in down regulating androgen receptor is complete (Task 2)

(Please see the results in next few pages; Figure 1-8)



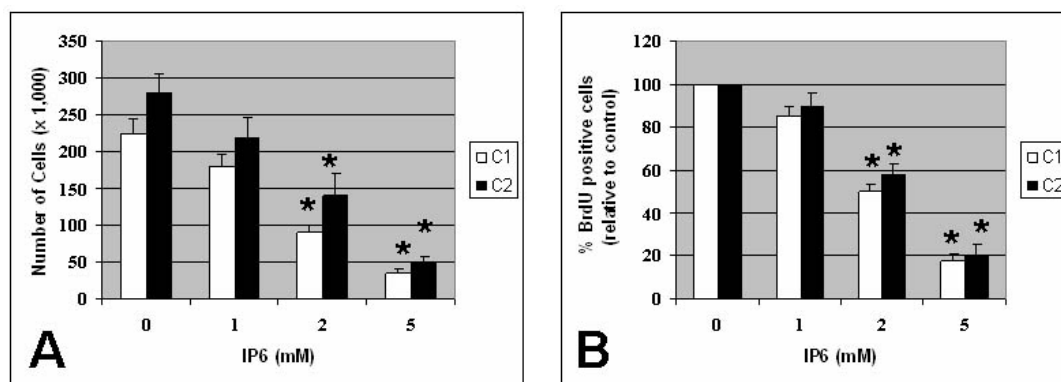
**Figure 1.** Effects of IP6 treatment on TRAMP prostate growth in vivo. A: Weight of prostate tumor after 32 weeks of IP6 treatment in vivo. IP6 induced a dose-dependent decrease in prostate tumor growth. B: a representative photograph from a control TRAMP tumor at 36 weeks of age. C: a representative photograph of a 4% IP6-treated TRAMP prostate. \* indicates significant differences from control. Data represents the results of 8 animals per group.

Although, we observed a dose-dependent inhibition of prostate tumor growth, these doses of IP6 did not cause any overt toxicity in these animals. As we observed, there is no significant change in the body weight (Fig 2A) or in five vital organs, heart, kidney, liver, lung and testis (Fig. 2B). We are currently looking into the histopathology of these organs to confirm that IP6 did not cause any damage to these vital organs over 8 months of treatment.



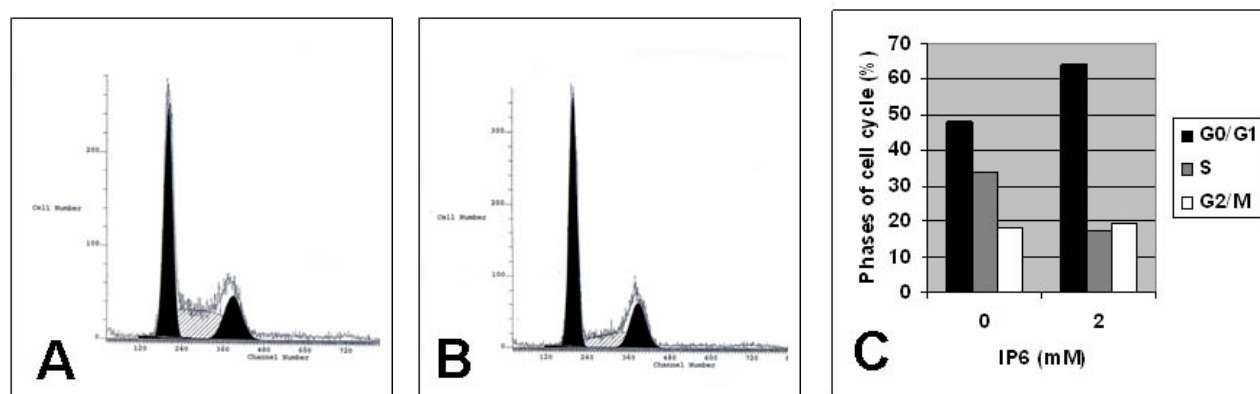
**Figure 2.** Effect of IP6 on body weight and organ weights in TRAMP mice. A: Body weights at 36 weeks-old TRAMP mice after various doses of IP6 treatment. B: Various organ weights at 36 weeks of age after various doses of IP6 treatment.

To examine the *in vivo* effect of IP6 at the molecular level, we examined the effect if IP6-induced growth inhibition in TRAMP cells. First, we examined whether IP6 inhibits TRAMP prostate cancer cells growth. As we see in Figure 3A there is a dose-dependent decrease in cell growth in both TRAMP C1 and C2 cells. Significant inhibition occurred by 2 mM of IP6. Similarly, we also observed that IP6 also decreased the DNA synthetic ability of these cells dose-dependently, and by 2 mM concentration BrdU labeling was decreased approximately 50% (Fig 2B), suggesting that similar to *in vivo* situation, IP6 can inhibit TRAMP prostate cancer cell growth *in vitro*.



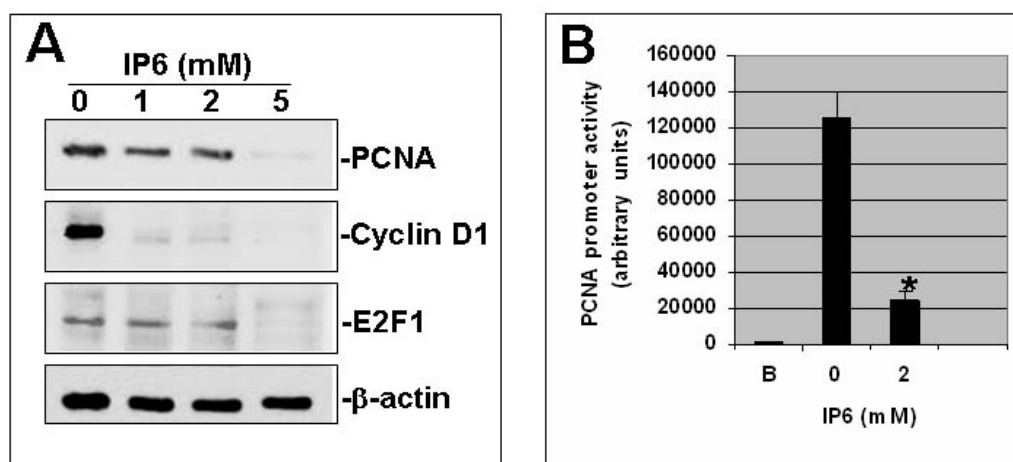
**Figure 3.** Effects of IP6 on growth and DNA synthesis of TRAMP cells in complete growth media. A: Dose-dependent growth inhibition of TRAMP C1 and C2 cells after 3 days of culture. B: Dose-dependent inhibition of DNA synthesis (BrdU incorporation) in TRAMP C1 and C2 cells after 3 days. \* indicates significant differences compared to their respective controls.

We also observed that IP6 induces G0/G1 arrest in TRAMP C2 cells as early as 24h of treatment (Fig 4). As a result the S-phase decreases significantly. It was also evident in earlier experiment in BrdU incorporation study (Fig. 3B).



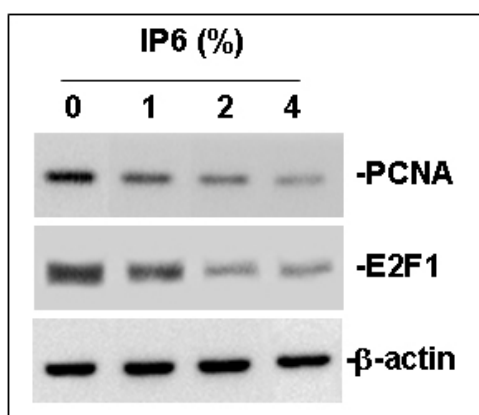
**Figure 4.** Effects of IP6 on cell cycle progression in TRAMP cells. A: Flow cytometric data showing various phases of cell cycle in TRAMP cells with (B) or without (A) IP6 treatment for 24h. C: quantitative data comparing the cell cycle between IP6 treatment and without treatment.

Since, we observed the cell cycle arrest at G0/G1, we decided to examine some of the dominant players of this phase of cell cycle. As we see in Figure 5A, the level of PCNA, cyclin D1 and E2F1 decreased dose-dependently. The dramatic effect was observed in cyclin D1, where even 1 mM IP6 caused almost complete inhibition of this protein expression. We are currently looking at the promoter of this gene to determine the molecular regulation of cyclin D1 by IP6. Using PCNA promoter-luciferase construct, we observed that IP6 induced a significant decrease in the PCNA promoter activity (Fig. 5B). This result suggests that IP6 can inhibit prostate cancer growth by down regulating the PCNA transcription and by decreasing PCNA protein expression. We do not know whether the translocation of PCNA is also associated with the IP6 treatment.



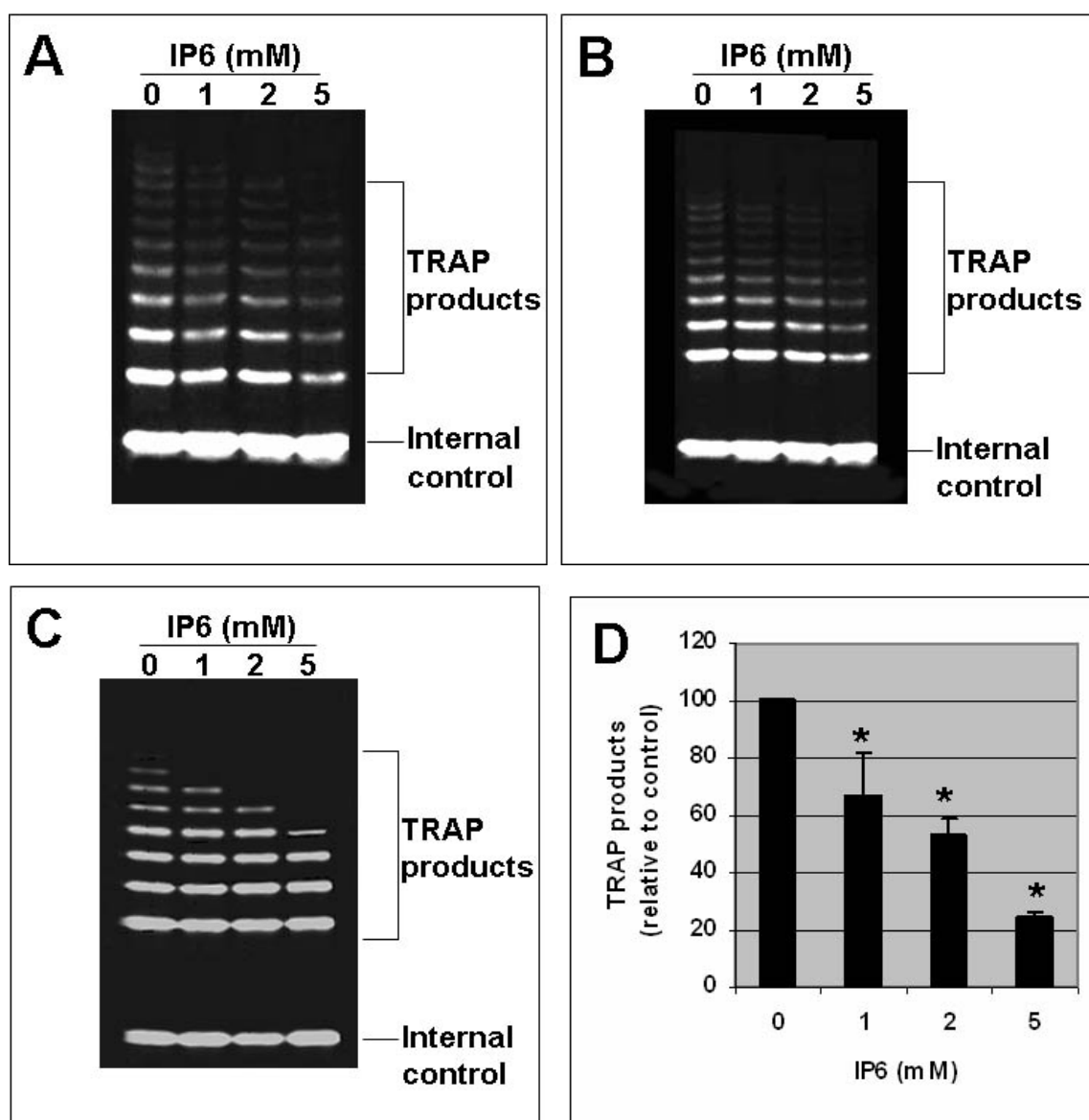
**Figure 5.** Effects of IP6 on cell cycle regulators of G0/G1 phase in TRAMP cells. A: Western blots showing the levels of PCNA, Cyclin D1 and E2F1 after various doses of IP6 treatment. B: PCNA promoter activity with or without IP6 treatment in TRAMP C2 cells. \* indicates significant differences compared to the respective control.

Similarly, in vivo studies also demonstrated a dose-dependent reduction in PCNA and E2F1 protein levels with IP6 treatments, suggesting that similar mechanisms operate in both in vivo and in vitro.



**Figure 6.** Effects of in vivo treatments of IP6 on PCNA and E2F1 in TRAMP prostate. Western blots showing the levels of PCNA, and E2F1 after various doses of IP6 treatment.

In search of molecular mechanisms of IP6 induced growth inhibition of TRAMP cancer cells, we discovered that IP6 dose-dependently decreased telomerase activity (Fig. 7). We also observed that this decrease in telomerase activity is not TRAMP cell specific, it also occurs in human prostate cancer cells, LNCaP (Fig. 7C). Using quantitative estimation, we observed that with 2 mM IP6 caused 50% inhibition of telomerase activity by 3 days of treatment and with 5 mM concentration it further reduces to approximately 20% of the control levels (Fig. 7D). These results clearly suggest for the first time that IP6 can inhibit telomerase activity in prostate cancer cells and thereby inhibits prostate cancer cells ability to replicate indefinitely.

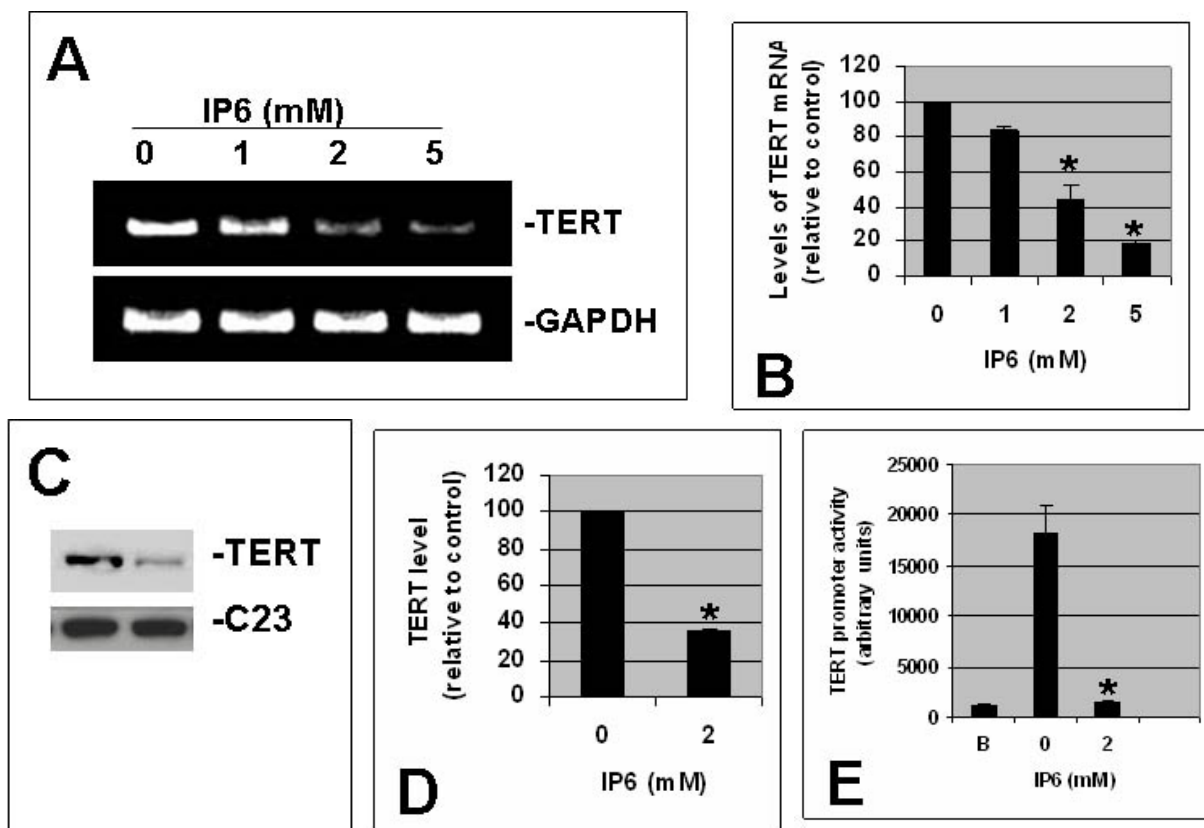


**Figure 7.** Effects of IP6 on the telomerase activity in TRAMP and human prostate cancer cells. A: TRAP assay showing the levels telomerase activity in IP6 treated and untreated TRAMP C1 cells. B: TRAP assay showing the levels telomerase activity in IP6 treated and untreated TRAMP C2 cells. C: TRAP assay showing the levels telomerase activity in IP6 treated and untreated LNCaP cells. D:



Quantitative estimation of telomerase activity with various doses of IP6 in TRAMP C2 cells. \* indicates significant differences compared to the respective control.

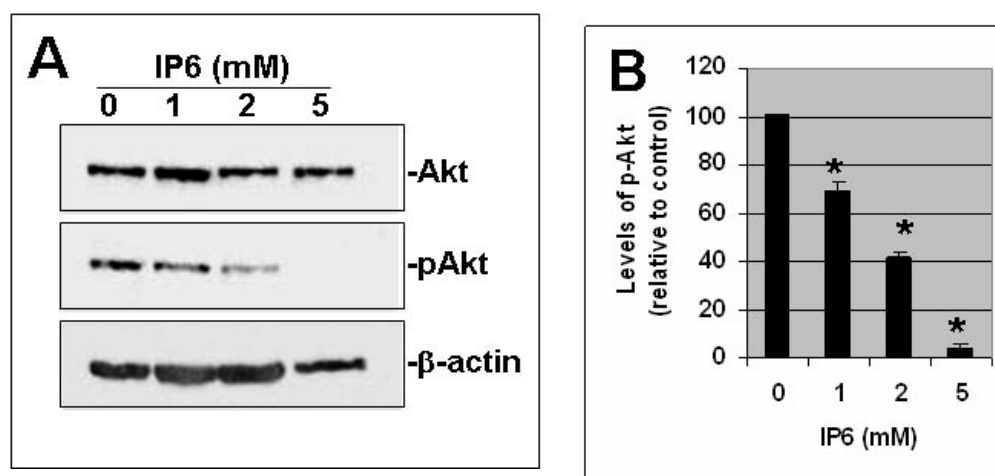
Since we observed that the telomerase activity decreases in response to IP6 treatment in TRAMP and human prostate cancer cells, we examined the message level of TERT, catalytic subunit of telomerase, expression of TERT is tightly associated with the telomerase activity. Using RT-PCR, we examined the mRNA level of TERT and normalized with GAPDH expression. As seen in Fig. 8A, there is a dose-dependent decrease in the expression of TERT mRNA, suggesting decrease in telomerase activity is associated with the decrease in TERT expression. Using quantitative estimation again we observed that 2 mM IP6 caused 50% decrease in TERT expression (Fig 8B). Because TERT is generally present in the nucleus, we examined the levels of TERT protein in the nuclear fraction using IP6 treated and untreated TRAMP C2 cells. As we see in Fig. 8C, TERT protein level decreased dramatically in the nuclear fraction. Quantitatively more than 60% of the protein was decreased after IP6 treatment (Fig 8D). We also examined the TERT promoter activity using a 3.3kb TERT promoter-luciferase construct. As we see in Figure 7E, TERT promoter activity was increased approximately 20-fold compared to the basic constructs and IP6 treatment decreased TERT promoter activity in TRAMP C2 cells almost 20-fold. These results again reconfirms our telomerase activity data and reemphasize that IP6 regulates telomerase activity.



**Figure 8.** Effects of IP6 on telomerase protein and message in TRAMP prostate cancer cells. A: RT-PCR showing the levels TERT mRNA in IP6 treated and untreated TRAMP C2 cells. B: Quantitative analysis of TERT mRNA in IP6 treated and untreated TRAMP C2 cells. C: Western blot (nuclear

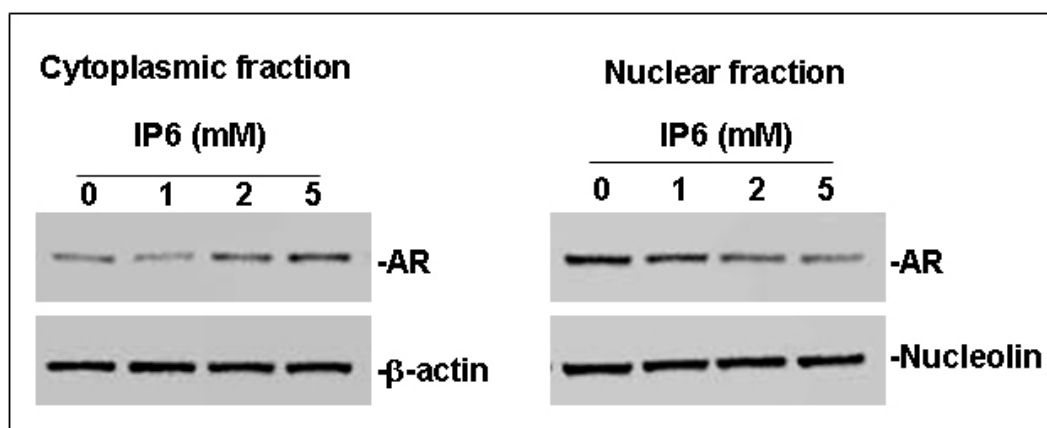
extract) showing the levels TERT protein in IP6 treated and untreated TRAMP C2 cells. C-23 (Nucleolin) was used as a loading control. D: Quantitative analysis of TERT protein after normalizing with C-23. E: TERT promoter activity in TRAMP C2 cells with or without IP6 (2mM) for 24h. \* indicates significant differences compared to their respective controls.

Since activation of telomerase requires phosphorylation of TERT and Akt is known to phosphorylate TERT, we examined the total and phosphorylated Akt with or without IP6 treatments. As we see in Figure 9, IP6 decreased the phospho-Akt but not the total Akt, suggesting that Akt is deactivated by the IP6. Quantitatively, we also observed that IP6 significantly decreased the activation of Akt (Fig. 9B). These results suggest that Akt is no longer able to phosphorylate TERT and therefore its translocation to the nucleus.



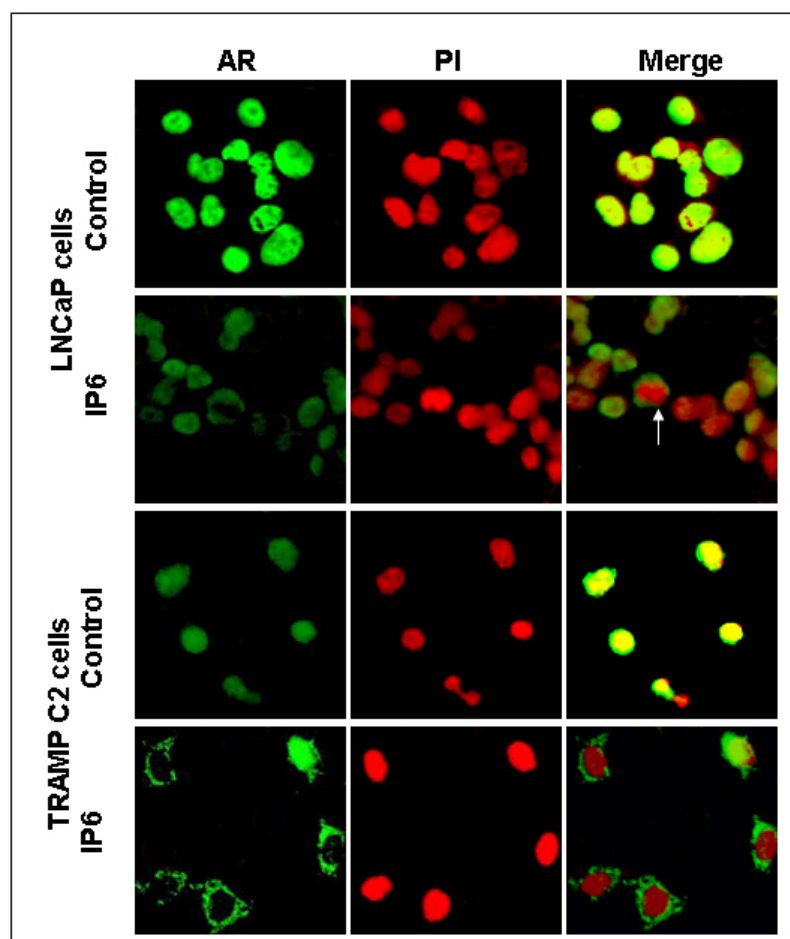
**Figure 9.** Effects of IP6 on the levels and activation of Akt in TRAMP cells. A: Western blot analyses of total and activated Akt after various doses of IP6 treatments. B: Quantitative analysis of activated Akt with various doses of IP6 in TRAMP C2 cells. \* indicates significant differences compared to the respective control.

As an additional mechanism, we also observed that IP6 dose-dependently decreased androgen receptor levels (data not shown) and prevent the translocation of androgen receptor in the nucleus. So, more androgen receptors are found in the cytoplasmic fractions compared to the nucleus (Fig. 10).



**Figure 10.** Effects of IP6 on the levels cytoplasmic and nuclear androgen receptor in LNCaP cells. Beta-actin and nucleolin were used as loading controls, respectively.

We observed the prevention of androgen receptor translocation in both LNCaP and TRAMP-C2 cells (Figure 11). As we see androgen receptor is localized in the nucleus in both LNCaP and TRAMP-C2 cells. Treatment of these cells with 2 mM IP6 for two days prevented translocation of androgen receptor to the nucleus, so some androgen receptor were in the cytoplasm (see the merge figures).



**Figure 11.** Effects of IP6 on the translocation of androgen receptor in LNCaP and TRAMP-C2 cells. AR: androgen receptor, PI: propium iodide.

**REPORTABLE OUTCOMES:** We already submitted one manuscripts entitled “Inositol hexaphosphate represses telomerase activity in mouse and human prostate cancer cells via the inactivation of Akt” (please see in appendices). Other two manuscripts are in preparation at this point. As soon as we hear the acceptance of these manuscripts, I will send preprints of these manuscripts to US Army Medical Research Material Command. We will also report these findings in upcoming AACR meeting.

**CONCLUSIONS:** We have completed our research on the effect of IP6 in prevention of prostate cancer and discovered that IP6 can prevent the development poorly differentiated prostate cancer in TRAMP model. Mechanistically, we observed that IP6 can repress telomerase activity in both mouse and human prostate cancer cells that could inhibit growth and survival pf prostate cancer cells. In addition, we also observed that IP6 can prevent the translocation of androgen receptor and thereby would inhibit androgen receptor dependent growth regulation. To understand the detailed mechanism, further investigation would be required.

**REFERENCES:** N/A

**APPENDICES:** Please see the manuscript that we have submitted in Cancer Research.

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# **Inositol hexaphosphate represses telomerase activity in mouse and human prostate cancer cells via the inactivation of Akt**

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Keywords: Inositol hexaphosphate, prostate cancer, telomerase, TERT, Akt

Running title: Inositol hexaphosphate inhibits telomerase activity

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## **Abstract**

Inositol hexaphosphate (IP6), the most abundant phosphorylated inositol present in most cereals, nuts, legumes and soybeans has anti-proliferative effects on a variety of cancer cells, including prostate cancer. However, the molecular mechanism of anti-proliferative effects of IP6 is not entirely understood. Since the activation of telomerase is crucial for cells to gain immortality and proliferation ability, we examined the role of IP6 in the regulation of telomerase activity in prostate cancer cells. Here we show that IP6 down-regulates TERT message and protein and thereby represses telomerase activity in mouse and human prostate cancer cells dose-dependently. To investigate the molecular mechanism of IP6-induced repression of telomerase activity in prostate cancer cells, we examined one of the key kinases, Akt, that activates TERT by phosphorylation at the serine residue. Activation of TERT is essential for the translocation of TERT to the nucleus and for full telomerase activity. Our data show that IP6 prevents the phosphorylation of Akt and thereby phosphorylation of TERT. As a result, IP6 prevents the translocation of TERT to the nucleus in both mouse and human prostate cancer cells. These results show for the first time that IP6 represses telomerase activity in prostate cancer cells by posttranslational modification of TERT via the Akt and strengthen its role as an anti-cancer agent.

## **Introduction**

Telomerase is a ribonucleoprotein enzyme with specialized reverse transcriptase activity that catalyses the synthesis and extension of telomeric DNA (1). This enzyme is present in germ line cells, cancer-derived cell lines, spontaneously immortalized cells in culture and is activated in 85%-90% malignant tumors but usually absent in normal somatic cells which results in the progressive loss of telomeres with each cell division (2). Cells require a mechanism to maintain telomere stability in order to overcome replicative senescence, and telomerase activation may therefore be a rate limiting step in cellular immortality and oncogenesis (3). The telomerase complex is composed of telomerase reverse transcriptase (TERT), telomerase RNA (TR), telomerase associated protein (TEP1), and chaperone proteins (p23, Hsp90) (4). A strong correlation is observed between TERT mRNA expression and telomerase activity in a variety of epithelial cancers (5). It has been demonstrated that Akt kinase enhances human telomerase activity through phosphorylation of the TERT subunit at the region surrounding Ser-824 (6). Recent evidence shows that telomerase modulates expression of growth controlling genes and enhances cell proliferation (7). Therefore, factors that are involved in the regulation of telomerase activity have generated considerable interest in recent years. Such factors have significant importance in understanding and manipulating cell growth in neoplasia.

Several epidemiological studies have pointed out that a high-fat and low-fiber diet, as consumed by most of the industrialized world, increases cancer risk, while plant-based diets, rich in whole grains, legumes, fruits and vegetables contains high fiber diet is associated with the reduction of various cancers including, breast, colon and prostate (8).



The active ingredients of cereals and legumes contain inositol hexaphosphate (IP6). IP6 constitutes 0.4-6.4% (w/w) of most cereals, nuts, legumes and soybeans (9). Elegant work by Shamsuddin and his associates (9-11) and others (12-14) have demonstrated a profound anti-cancer effect of IP6 against colon, mammary, lung, liver, leukemia, skin as well as prostate cancer. Mechanistically, IP6 alters PI3 kinase activity (12), up regulates tumor suppressor genes (15), and inhibits angiogenesis (16). These studies suggest that IP6 acts on various targets to inhibit cancer cell growth. Since some recent evidence shows that telomerase modulates expression of growth controlling genes and enhances cell proliferation (7) we hypothesized that IP6 could inhibit telomerase activity.

Using mouse and human prostate cancer cell lines, TRAMP-C2 and DU-145, we show for the first time that IP6 inhibits telomerase activity by the posttranslational modification of TERT via the deactivation of Akt.

## **Materials and Methods**

**Reagents and cell culture.** Antibodies against total Akt, phospho-Akt Ser473 and phospho-serine antibody were purchased from Cell Signaling, Beverly, MA. TERT antibody was purchased from Novocastra Laboratories, Newcastle upon Tyne, UK. Protein A/G-Sepharose beads and nucleolin (C23) antibody were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA.

TRAMP-C2 (a gift from Dr. Norman Greenberg, Fred Hutchinson Cancer Research Center) and DU-145 (American Type Culture Collection, Manassas, VA) were grown in IMEM without phenol red (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Quality Biologicals, Gaithersburg, MD), 2mM glutamine, 100

units/ml penicillin G sodium and 100µg/ml streptomycin sulfate (Sigma, St. Louis, MO) in the presence of 5% CO<sub>2</sub> at 37°C.

**Telomeric repeat amplification protocol (TRAP) assay.** A cellular extract was prepared and the TRAP assay was performed according to our previously published method (17). Detection of telomerase activity in cell extracts was performed in a two step process: 1) telomerase-mediated extension of an oligonucleotides (TS: 5'-AATCCGTCGAGCAGAGTT-3'), and 2) PCR amplification of the resultant product with forward (TS) and reverse (ACX: 5'-GCGCGGCTTACCCTTACCC TTACCCTAACC-3') primers. In addition, as internal control, NT: 5'-ATCGCTTCTCGGCCTTTT-3'; TSNT: 5'-AATCCGTCGAGCAGAGTTAAAAGGCC GAGAAGCGAT-3' primers were used. Telomeric DNA products were separated by 10% nondenaturing PAGE in 0.5 x Tris-borate-EDTA buffer, pH 8.3, at 300V for 2.5h. Gels were then stained with SYBR Green I and image was captured with Fuji LAS-1000 imager. TRAP products were quantified by densitometric analysis using NIH Image J program (NIH, Bethesda, MD).

**Isolation of total RNA and reverse transcriptase polymerase chain reaction (RT-PCR).** DU-145 and PC3 cells were plated and cultured as described above. RNA was extracted with TRIzol solution as suggested by the manufacturer (Invitrogen, Carlsbad, CA). Genes of interest were amplified using 1 µg of total RNA reverse transcribed to cDNA using Superscript II kit (Invitrogen) with random hexamers. Mouse specific primers were designed by us using the Primer Quest program and purchased from IDT (Coralville, IA). mTERT-F: 5'-ACTCAGCAACCTCCAGCCTA-3', mTERT-R: 5'-CATATTGGCACTCTGCATGG-3'; mTEP1-F: 5'-GAGCACCTTGGAGCAAGAAC-

3', mTEP1-R: 5'-CTTCTCTAGCCCCAGCCTTT-3', and mGAPDH-F: 5'-GTGTTCTACCCCCAATGTG-3', mGAPDH-R: 5'-CTTGCTCAGTGTCCTTGCTG-3'. PCR reaction was initiated at 94°C for 1 minute, followed by 30 cycles of 94°C for 1 minute, 1 minute annealing temperature, 72°C for 1 minute followed by final extension at 72°C for 5 minute. Number of cycle used to amplify GAPDH was 26. Annealing temperatures for mouse TERT, TEP1 and GAPDH were 57°C, 56°C and 60°C, respectively and the yielded PCR products (179, 337, 349 base pairs, respectively) were separated on 1.5% agarose gels and visualized by ethidium bromide fluorescence using the Fuji LAS-1000 imager (Tokyo, Japan). Images were captured and imported to Photoshop. Information about the human specific primers for TERT, TEP1 and GAPDH (yielded PCR products of 145, 280, 598 base pairs, respectively) and PCR conditions were described previously (17).

**Preparation of nuclear extracts, immunoprecipitation and immunoblot.** The preparation of nuclear extracts was performed using the nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Immunoprecipitation was performed as we described earlier. For routine immunoblot protein extracts were prepared from TRAMP-C2 and DU-145 cells treated with or without IP6 according to our previously published methods (17). Membranes were probed with specific antibodies against proteins of interest then each membrane was stripped and re-probed with  $\beta$ -actin antibody (Sigma) to ensure for equal loading. Molecular weight markers (Invitrogen) were run on each gel to confirm the molecular size of the immunoreactive proteins.

**Immunocytochemical staining.** TRAMP-C2 and DU-145 cells were plated on ECL-coated (Upstate Biotechnology, Lake Placid, NY) Lab-Tek chamber slide. After 24 hours of treatment with IP6 or Wortmannin cells were fixed in chilled methanol at -20°C for 30 minutes, blocked with 1% BSA at room temperature for 1 hour then probed with TERT antibody (Novocastra Laboratories) overnight at 4°C followed by incubation with the Alexa Fluor 488 conjugated anti-mouse antibody (Molecular Probes, Inc., Eugene, OR) for 1 hour. Cells were counterstained with propidium iodide. Slides were viewed under a fluorescent microscope (ZEISS AxioPlan2 Imaging System, Jena, Germany). Captured images were imported to Photoshop.

**Statistical analyses.** Data from the TRAP assays, Western blots and RT-PCRs were derived from at least 3 independent experiments. Statistical analyses were conducted using the Prism3 GraphPad software and values were presented as mean  $\pm$  SEM. Significance level was calculated using the one-way Analysis of Variance (ANOVA) followed by the Dunnett post-test, with an assigned confidence interval of 95%. P value < 0.05 was considered significant.

## **Results**

**IP6 represses telomerase activity in prostate cancer cells.** Since IP6 has anti-proliferative effects on various cancer cells, and the majority of cancer cells have high telomerase activity that provides cancer cells with the ability to survive and proliferate, we examined whether IP6 represses telomerase activity in prostate cancer cells. Using the TRAP assay, we observed that IP6 repressed telomerase activity in both mouse (TRAMP-C2) and human (DU-145) prostate cancer cells dose-dependently (Fig. 1A and

B). As little as 1 mM IP6 repressed telomerase activity in TRAMP-C2 cells significantly ( $p < 0.01$ ) (Fig. 1C) but significant repression was not observed in DU-145 cells at this concentration (Fig. 1D). However, 2 and 5 mM of IP6 repressed telomerase activity (~50%) and (~80%) in both TRAMP-C2 and DU-145 cells, respectively (Fig. 1C and D). These concentrations of IP6 (1 and 2 mM) did not induce cell death in these prostate cancer cells (data not shown). These results suggest that IP6 has an inhibitory effect on telomerase activity in prostate cancer cells.

#### **IP6 down-regulates TERT expression but not telomerase associated protein, TEP1.**

Expression of the TERT is tightly regulated with telomerase activity in various cancer cells, therefore, it is important to examine whether the inhibitory effect of IP6 on telomerase activity in prostate cancer cells is due to the down-regulation of TERT expression. We observed that IP6 treatment for 3 days decreased TERT expression in TRAMP-C2 (Fig. 2A) and DU-145 (Fig. 2B) dose-dependently, however, the expression of telomerase associated protein, TEP1, did not change (Fig. 2A and B, left panels). With increasing concentrations of IP6, TERT message levels were decreased dose-dependently and significantly ( $p < 0.01$ ) (Fig. 2A and B, right panels), and 2 mM of IP6 decreased approximately 50% of TERT message. Similarly, TERT protein levels in TRAMP-C2 and DU-145 cells were also decreased significantly with IP6 treatments (Fig. 2C). These results suggest that repression of telomerase activity in TRAMP-C2 and DU-145 cells with IP6 treatment is due to the IP6-dependent down-regulation of TERT expression.

**IP6 deactivates Akt in prostate cancer cells and deactivation of Akt is involved in repression of telomerase activity.** Because Akt enhances human telomerase activity

through phosphorylation of TERT (6), we investigated whether the repression of telomerase activity by IP6 was due to the deactivation of Akt. As seen in figure 3A, total Akt level did not change with various doses of IP6 treatment of TRAMP-C2 cells, whereas phospho-Akt Ser473 residue was decreased dose-dependently. Quantitatively, phospho-Akt Ser473 was decreased significantly ( $p < 0.01$ ) with as little as 1 mM concentration of IP6 and with 2 mM more than 50% of phospho-Akt Ser473 was decreased. Similar results were observed in DU-145 cells, suggesting that deactivation of Akt is common phenomenon in mouse and human prostate cancer cells. These results suggest that IP6 deactivates Akt kinase by decreasing phosphorylation of Ser473.

**IP6 prevents phosphorylation of TERT and thereby inhibits translocation to the nucleus in prostate cancer cells.** Since activated Akt phosphorylates TERT and it is necessary for full telomerase activity, we examined whether phosphorylation of TERT is inhibited by the IP6 treatment. Immunoprecipitation immunoblot analyses demonstrate that 2 mM of IP6 decreased the phospho-TERT in DU-145 (Fig. 3B) and TRAMP-C2 (data not shown). Approximately 3-fold decrease in phospho-hTERT was observed in IP6-treated DU-145 cells compared to the untreated control cells. Since we believe that deactivation of Akt is one of the causes of repressed telomerase activity by IP6, we used PI3 kinase inhibitor, Wortmannin to deactivate Akt in DU-145 cells. We observed that 50 nM Wortmannin treatment for 24 hours is enough to significantly deactivate Akt (Ser473) without altering total Akt (Fig. 3C). Fifty nanomolar of Wortmannin decreased phosphorylation of TERT 2.5-fold and significantly decreased ( $p < 0.01$ ) the telomerase activity compared to the untreated control cells (Fig. 3D). These results suggest that deactivation of Akt prevents phosphorylation of TERT and thereby represses telomerase

activity.

Since phosphorylation of TERT is necessary for its nuclear translocation and complete telomerase activity, we subsequently examined the localization of TERT protein in DU-145 and TRAMP-C2 cells with or without IP6 and Wortmannin treatments. As seen in figure 4, in untreated control cells TERT protein is localized predominantly in the nucleus. Treatments of DU-145 cells with IP6 (2 mM) or Wortmannin (50 nM) decreased nuclear TERT protein staining intensity accompanied by an increase in cytoplasmic staining. The translocation of TERT by the treatments with IP6 or Wortmannin was more pronounced in the case of TRAMP-C2 cells. These results suggest that similar to Wortmannin, IP6 decreased phosphorylation of TERT via the deactivation of Akt and thereby inhibited its translocation to the nucleus.

## **Discussion**

In the present study, we investigated the role of IP6 in the regulation of telomerase activity in prostate cancer cells. Using mouse and human prostate cancer cell lines, TRAMP-C2 and DU-145, we demonstrated that IP6 dose-dependently (1-5 mM) represses telomerase activity. We show that IP6-induced repression of telomerase activity is due to the posttranslational modification of TERT protein via Akt.

Posttranslational modifications are important for full telomerase activity. TERT is a phosphoprotein and its activity is modulated by a complex set of protein kinases, providing posttranslational control on telomerase regulation. It has been shown that protein kinase B (PKB/Akt) is involved in phosphorylation of TERT protein and the up-regulation of telomerase activity (6). Kang et al demonstrated that the serine residue at position 824 of TERT was phosphorylated by the Akt (6). In the present study, we

observed that IP6 treatment down-regulated phospho-Akt Ser473 dose-dependently in TRAMP-C2 and DU-145 cells without affecting the total Akt (Fig. 3). Moreover, phosphorylation of TERT was also decreased significantly after IP6 or Wortmannin treatments (Fig. 3). Our result corroborate with an earlier study in melanoma cells where TERT peptide phosphorylation and telomerase activity were down-regulated by the Wortmannin treatment (6). These results clearly suggest that IP6 acts at the posttranslational level to down-regulate phosphorylation of TERT via the Akt pathway. Deactivation of Akt by IP6 treatment has been demonstrated by earlier study (18). Beside Akt, PKC, another serine/threonine kinase is also know to be involved in phosphorylation of TERT and thereby telomerase activity (19). It is possible that in addition to the deactivation of Akt, IP6 might also inhibit PKC and thereby suppress telomerase activity. Future studies will be necessary to determine the whether PKC is involved in IP6-induced dephosphorylation of TERT.

It is well documented that cellular localization of TERT could be a potential mechanism of telomerase activation through TERT phosphorylation linked to nuclear localization (20). Nuclear translocation of TERT from a presumably non-functional cytosolic location to a physiologically relevant nuclear compartment may be one of the mechanisms underlying the regulation of the telomerase function in cells. It was previously reported that 14-3-3 proteins bind to TERT and increase its nuclear localization (21). Similarly, NFkB p65 is also a post-translational modifier of telomerase which is involved in the intracellular localization of TERT (22). Using immunofluorescent staining, we demonstrated that IP6 treatment decreased the nuclear staining for TERT and the residual staining was observed in the cytoplasm (Fig. 4), suggesting that IP6 decreased the phosphorylation of TERT and thereby it was unable to



bind its nuclear translocator or decreased phosphorylation of TERT forcing it to translocate from the nucleus to the cytoplasm. Treatment with Wortmannin had a similar effect on TERT translocation suggesting that the Akt deactivation by IP6 would be sufficient to decrease phosphorylation of TERT. However, it is also possible that IP6 might down-regulate NFkB and/or 14-3-3 protein levels in addition to the dephosphorylation of Akt and thereby decrease their association with TERT and eventually inhibits translocation to the nucleus. In fact, it has been demonstrated that IP6 inhibits constitutive activation of NFkB in prostate cancer cells (23). It is also possible that in addition to posttranslational modification, IP6 could also alter transcriptional activity of TERT. We observed a dose-dependent down-regulation of TERT message but we do not know at this point whether down-regulation of TERT is due to the transcriptional repression of TERT. We postulate that the deactivation of Akt will increase GSK3 $\alpha/\beta$  activity and eventually degrade c-Myc protein. Because of the paramount importance of c-Myc in TERT transcriptional activation (24), it is possible the down-regulation of c-Myc would repress telomerase activity. Future studies will be necessary to define the role of c-Myc in IP6-dependent repression of telomerase activity.

In summary, this study demonstrates for the first time that IP6 represses telomerase activity in cancer cells. Down-regulation of TERT message as well as the posttranslational modifications of TERT protein is involved in IP6-induced repression of telomerase activity in prostate cancer cells. Repression of telomerase activity by IP6 strengthens the existing belief that IP6 could be a potential chemotherapeutic agent for various cancers, including prostate cancer.

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## Figure legends

**Figure 1.** IP6 represses telomerase activity in prostate cancer cells. A and B: Effect of IP6 on the level of telomerase activity in TRAMP-C2 and DU-145 cells, respectively. Twenty-four hours after plating, cells were exposed to various concentrations of IP6 (0, 1, 2 and 5 mM) for 3 days. Cell pellets were collected and subjected to telomere repeat amplification protocol (TRAP) assay. NC: Negative control using lysis buffer only. C and D: Quantitative estimations of telomerase activity in TRAMP-C2 (C) and DU-145 (D) cells determined by densitometric measurements of TRAP products from three independent experiments. Quantitative values are the mean  $\pm$  SEM. \*, indicates significantly different from control ( $p < 0.01$ ).

**Figure 2.** IP6 down-regulates TERT expression but not telomerase associated protein (TEP1) in prostate cancer cells. A and B: After 24h of plating, TRAMP-C2 (A) and DU-145 (B) cells were exposed to various concentrations of IP6 (0, 1, 2 and 5 mM). After 3 days of treatment, total RNA was extracted and RT-PCR assays were performed to detect TERT, TEP1 and GAPDH mRNAs. A representative photograph from an experiment that was repeated three times is shown on the left. Quantitative estimations of TERT mRNA in TRAMP-C2 and DU-145 cells were determined by densitometric measurements of RT-PCR gels from three independent experiments after normalization with GAPDH (shown on right panels). C: Western blot analyses showing TERT protein in TRAMP-C2 and DU-145 cells with (2 mM) or without IP6 treatments (left panels). Quantitative estimations of TERT protein levels are shown on right panel. Quantitative values represent mean  $\pm$  SEM. \*, indicates significantly different from control ( $p < 0.01$ ).

**Figure 3.** IP6 inhibits phosphorylation of Akt and thereby prevents phosphorylation of TERT in prostate cancer cells. A: Fifty micrograms of protein lysates from TRAMP-C2 and DU-145 cells treated with various concentrations of IP6 (0, 1, 2 and 5 mM) for 3 days were resolved on 12% SDS-PAGE and immunoblots were probed with antibodies to total Akt and phospho-Akt Ser473. All immunoblots were re-probed with  $\beta$ -actin antibodies to ensure for equal loading. A representative photograph from an experiment that was repeated three times is shown (left panels). Quantitative analyses of relative levels of phospho-Akt Ser473 are shown on right panels. Values are the mean  $\pm$  SEM from three independent immunoblots. \*, indicates significantly different from levels in untreated cells ( $p < 0.01$ ). B: IP6 dephosphorylates TERT in prostate cancer cells. DU-145 cells treated with (2 mM) or without IP6 for 3 days and then cells were harvested and nuclear fractions were immunoprecipitated with TERT antibody. Immunoprecipitated proteins were resolved on 12% SDS-PAGE and immunoblots were probed with phospho-serine antibody. Quantitative analyses of relative levels of phospho-TERT in the nucleus are shown below the immunoblot. Values are the mean of two independent experiments. C: Western blot analysis showing dose-dependent decrease in phospho-Akt Ser473 in DU-145 cells after 24 hours of Wortmannin treatment. D: TERT immunoprecipitated immunoblot showing the levels of phospho-TERT with (50 nM) or without Wortmannin treatment of DU-145 cells (left). TRAP assay showing the telomerase activity (TRAP products in middle panel and quantitative analysis on right panel) with (50 nM) or without Wortmannin treatment.

**Figure 4.** IP6 and Wortmannin treatments prevented the translocation of TERT to the nucleus in prostate cancer cells. DU-145 and TRAMP-C2 cells were plated on chamber slides 24 hours prior to the treatments with or without IP6 (2 mM) or Wortmannin (50 nM) for another 24 hours, then fixed in chilled methanol, incubated with TERT antibody and counter stained with propidium iodide. Slides were then mounted and examined under a fluorescence microscope. Photographs were taken at the same magnification (20X) merged and then imported to Photoshop. White arrow heads showing localization of TERT at the cytoplasm.

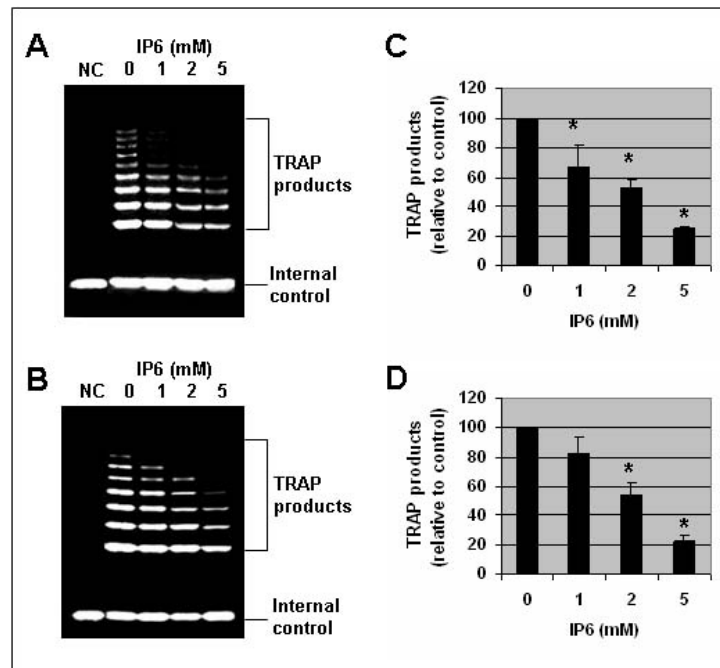


Figure 1. Jagadeesh and Banerjee



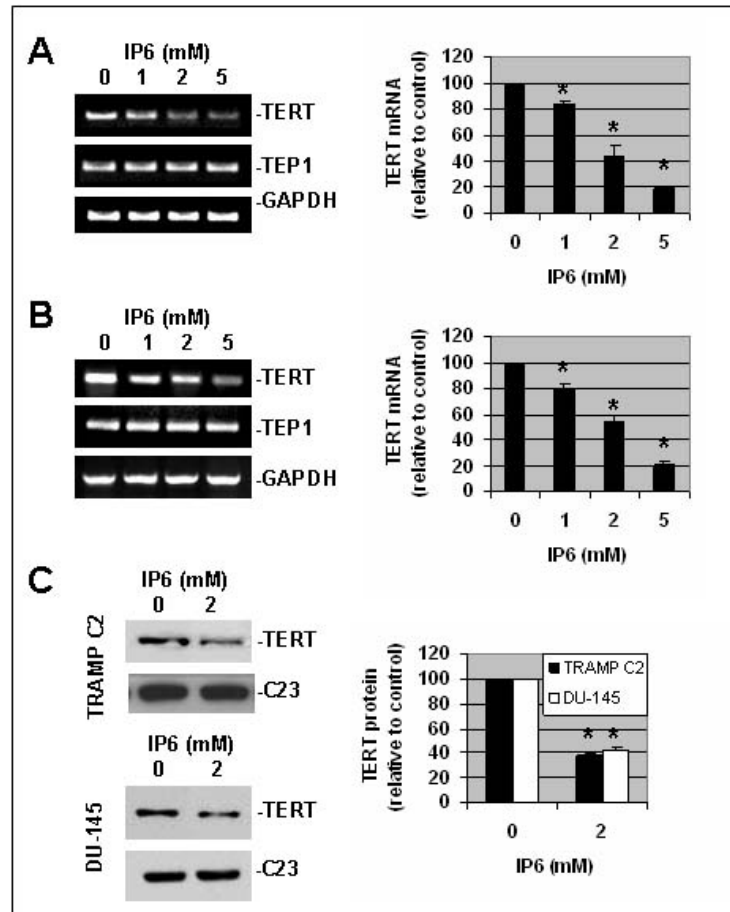


Figure 2. Jagadeesh and Banerjee

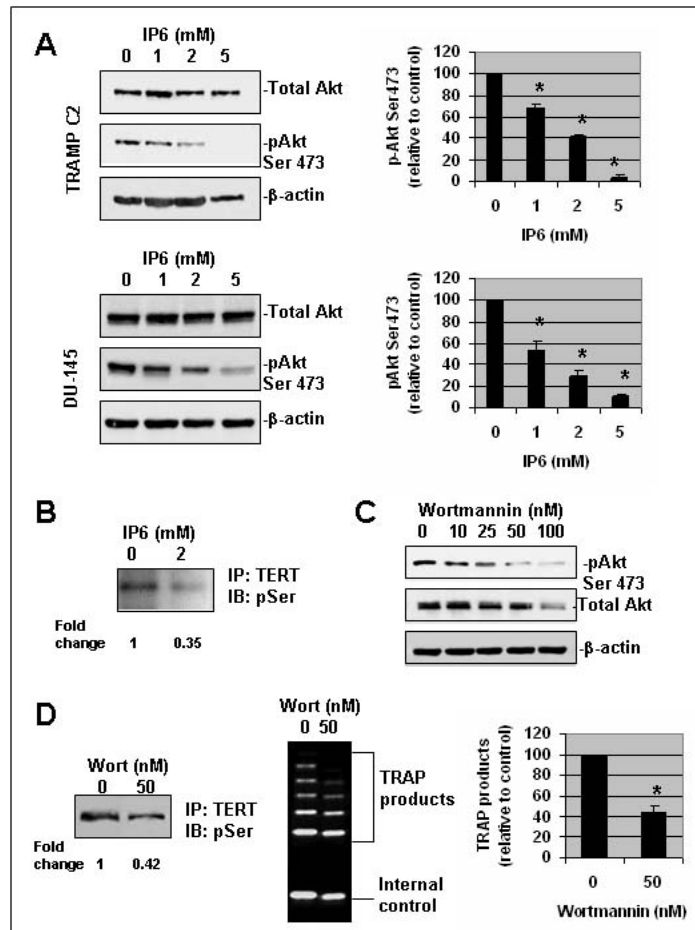


Figure 3. Jagadeesh and Banerjee

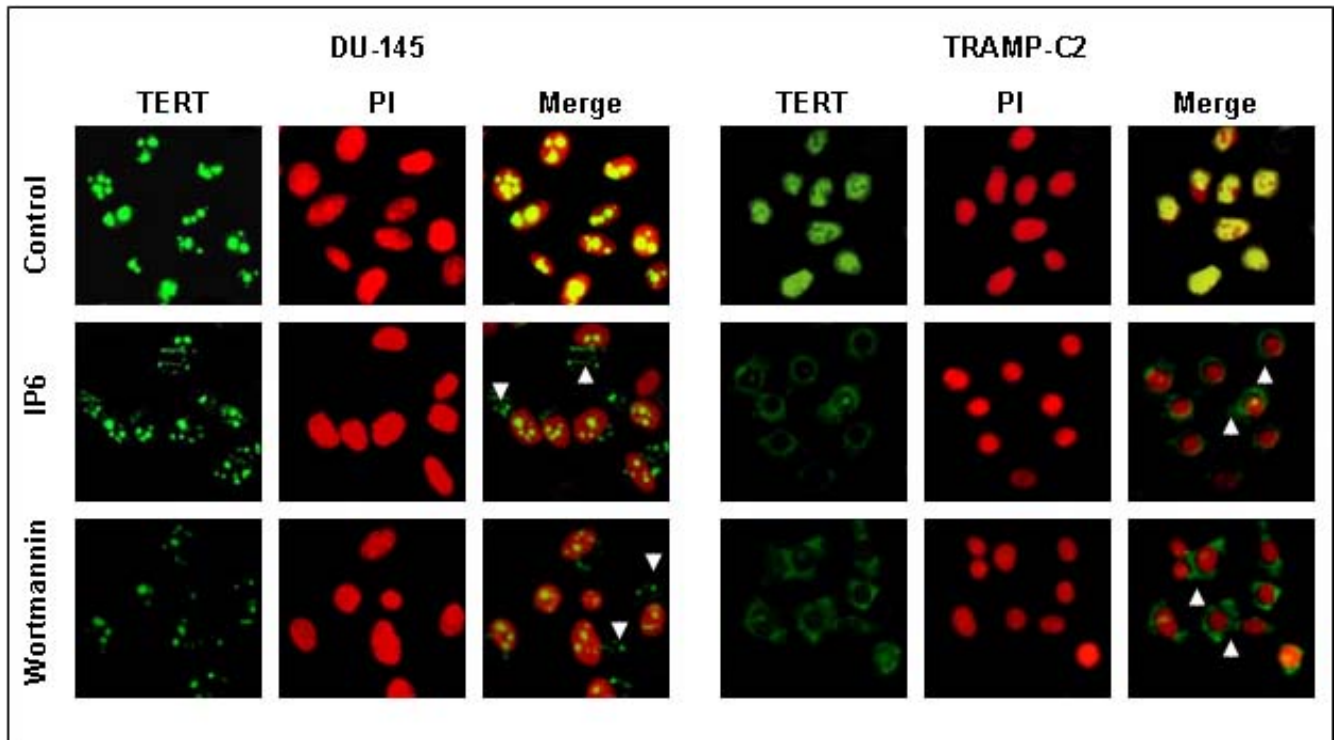


Figure 4. Jagadeesh and Banerjee