
Rana P. Singh1, Chapla Agarwal1 and Rajesh Agarwal1,3

1Department of Pharmaceutical Sciences, School of Pharmacy and  
2University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, CO 80262, USA  
3To whom correspondence should be addressed  
Email: Rajesh.Agarwal@UCHSC.edu

Carcinogenesis vol.24 no.3 pp.555–563, 2003

Introduction

Prostate cancer (PCA) is the major non-skin cancer in elderly men and second most common male malignancy in the United States (1). Similar to other epithelial malignancies, autocrine and paracrine growth factor-receptor interactions and associated mitogenic signalling are the major contributors to deregulated PCA cell growth (2,3). Since, in advanced stage, PCA growth and development become androgen independent that renders androgen ablation therapy ineffective (4), in this situation, control of PCA through chemoprevention and intervention strategies is highly desirable. Cancer chemoprevention is the use of natural or pharmacologic agents to prevent the development of invasive malignancy by inhibiting early stages of carcinogenesis and suppression or reversal of premalignant lesions (5,6). Several epidemiological and laboratory studies have shown that many vegetables, fruits and grains as well as phytochemicals from non-dietary sources offer significant protection against various cancers (3,5,6).

Inositol hexaphosphate (IP6) is a natural dietary ingredient and constitutes 0.4–6.4% (w/w) of most cereals, legumes, nuts, oil seeds and soybean (7). IP6 is abundant in cereals and legumes, the consumption of which has been associated with reduction in mammary, colon and prostate cancer (7). Recent studies have reported anti-cancer effects of IP6 in various cancer models, both in vitro and in vivo (7–9). IP6 has been shown to: (i) inhibit DMBA-induced rat mammary cancer and human breast cancer cell growth (9,10); (ii) suppress large intestinal cancer in rats (8); (iii) inhibit growth and reverse transformed phenotype of HepG2 liver cancer cells (11), and regress liver cancer xenotransplant (12); (iv) prevent pulmonary adenomas in mice (13); and (v) prevent skin tumorigenesis (14), inhibit growth of mouse fibrosarcoma FSA-1 tumor xenografts (15), and inhibit cell transformation in mouse epidermal JB6 cells (16). However, limited studies are done with IP6 in PCA, and that molecular mechanisms of its anti-cancer effect are not well defined. Shamsuddin and Yang (17) showed that IP6 inhibits growth and induces differentiation of human PCA PC-3 cells. Recently, we showed that IP6 impairs erbB1 receptor and fluid-phase endocytosis, and associated mitogenic signalling as its growth inhibitory effect in human PCA DU145 cells (18).

The high expression of growth factor receptors (e.g. members of erbB family) and associated ligands, and their interactions are known to cause autocrine and paracrine loops for both mitogenic and anti-apoptotic signalling leading to uncontrolled growth and metastasis of PCA (19–21). The activation of these signalling cascades alters the activation of several cell cycle regulators including cyclin-dependent kinases (CDKs), cyclins, CDK inhibitors (CDKIs), retinoblastoma protein (pRb) and pRb-related proteins such as pRb/p107 and pRb2/p130, sub-sequently resulting in activation of E2F family of transcription factors commanding cell growth, proliferation, and cell survival (22–24). CDKIs are believed to play a critical role in cyclin-CDK-CDK1 complex and negatively regulate cell cycle progression from one stage to another (22). Furthermore, unphosphorylated forms of pRb and pRb-related proteins have been shown to inhibit cell proliferation by sequestering E2F family of transcription factors (24).

Abbreviations: CDKs, cyclin-dependent kinases; CDKIs, cyclin-dependent kinase inhibitors; IP6, inositol hexaphosphate; FACS, fluorescence-activated cell sorting; PARP, poly (ADP-ribose) polymerase; PCA, prostate cancer; PI, propidium iodide; pRb/p107, retinoblastoma-related protein/p107; pRb2/p130, retinoblastoma-related protein 2/p130.
5000 cells/cm² were plated in 60 mm dishes and next day, cells were fed assessing the effect of IP6 on exponentially growing DU145 cell growth, and 6; cyclin D1, D3, E and A; pRb/p107, pRb2/p130, E2F3, 4 and 5, and antibodies were from Neomarkers (Fremont, CA). Antibodies to CDK2, 4 from Life Technologies, Inc. (Gaithersburg, MD). Anti-Cip1/p21 antibody Chemical Co. (St Louis, MO). RPMI 1640 and other culture materials were –

Cell culture and IP6 treatment

DU145 cells were plated at 5000 cells/cm² in 60 mm dishes under the standard culture conditions detailed earlier and after 24 h, fed with fresh medium and treated with different concentrations (0.25–4 mM) of IP6. After 12, 24, 48 and 72 h, both floating and attached cells were collected by a brief trypsinization, and counted in duplicate with a hemocytometer. Each treatment and time point had three independent plates. The representative data shown in this study were reproducible in three independent experiments.

Flow cytometry analysis for cell cycle distribution

DU145 cells were grown in serum supplemented RPMI 1640 medium. At ~30% confluence, cells were treated with 0.25–2 mM IP6 for 12–72 h and at the end of each treatment time, cells were collected after a brief incubation with trypsin-EDTA followed by processing for cell cycle analysis. Briefly, 0.5 × 10⁶ cells were suspended in 0.5 ml of saponin/PI solution [0.3% saponin (w/v), 25 µg/ml PI (w/v), 0.1 mM EDTA and 10 µg/ml RNase (w/v) in PBS], and incubated overnight at 4°C in dark. Cell cycle distribution was then analyzed by flow cytometry using FACS core facility of University of Colorado Cancer Center. Finally, percentage of cells in different phases of cell cycle was determined by ModFit LT cell cycle analysis software.

Western blotting

For cell cycle regulatory molecules CDKs, CDKIs and cyclins, pRb-related proteins and E2Fs, 40–80 mg of protein lysates per sample were denatured in 2X SDS-PAGE sample buffer and subjected to SDS–PAGE on 8 or 12% Tris–glycine gel. The separated proteins were transferred on to nitrocellulose membrane followed by blocking with 5% non-fat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C. Membranes were probed for the protein levels of Cip1/p21, Kip1/p27, CDK2, CDK4, CDK6, cyclin D1, D2, D3, E and A, pRb/p107, pRb2/p130, E2F3, 4 and 5, PARP, caspase and β-actin using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody, and visualized by ECL detection system.

Cyclin-CDKI and pRb-related protein-E2F binding studies

For binding study, DU145 cells were treated with 2 mM IP6 for 24 h and whole cell lysates were prepared as detailed above. 200 µg protein lysates were cleared with protein A/G plus agarose beads (Santa Cruz) for 45 min at 4°C. Cyclin D1 and E were immunoprecipitated from protein lysates, using specific antibodies (2 µg) for 6 h incubation followed by addition of 25 µl of protein A/G plus agarose beads and rocking overnight at 4°C. Non-specific antiserum was also used as a control to observe the non-specific immunoprecipitation in similar conditions. Immunoprecipitates were washed three times with lysis buffer, and samples were boiled in 2X sample buffer for 5 min followed by centrifugation. The resulting clear supernatants were subjected to SDS–PAGE on 12% gel. The separated proteins were electrophoretically transferred on to nitrocellulose membrane followed by blocking with 5% non-fat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Membrane was probed and visualized for Cip1/p21, Kip1/p27, cyclin D1 and E as detailed above. Similarly, pRb/p107 and pRb2/p130 were immunoprecipitated from 400 mg protein lysates using specific antibodies and subjected to SDS-PAGE and western blotting, and membranes were probed with E2F3, 4 and 5 antibodies and visualized by ECL detection system.

Kinase assays

CDK2 and cyclin E-associated H1 histone kinase activity was determined as described by us recently (25) with some modifications. Briefly, 200 µg of protein lysates from each sample was pre-cleared with protein A/G plus agarose beads and CDK2 protein was immunoprecipitated using anti-CDK2 and cyclin E antibodies (2 µg) and protein A/G plus agarose beads. Beads were washed three times with lysis buffer and finally once with kinase assay buffer (50 mM Tris–HCl, pH 7.4, 10 mM MgCl₂ and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 30 µl of ‘hot’ kinase solution [0.25 µl (2.5 µg) of histone H1, 0.5 µl (5 mM) of [γ-32P]ATP, 0.5 µl of 0.1 mM ATP and 2.875 µl of kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS–PAGE, and protein bands were detected on autoradiography. Similarly, the CDK4-6 and cyclin D1-associated Rb kinase activities, CDK4, 6 and cyclin D1 proteins were immunoprecipitated using specific antibodies, and beads conjugated with antibody and proteins were washed three times with RB-lysis
Fig. 2. Effect of IP6 on cell cycle progression in DU145 cells. Cells were cultured in RPMI 1640 supplemented with 10% FBS, and treated with either vehicle or 0.25–4 mM concentrations of IP6. After 24 h of these treatments, cells were collected, washed with PBS, digested with RNase and then cellular DNA stained with propidium iodide as detailed in ‘Materials and methods’. Flow cytometric analysis was then performed for cell cycle distribution. The percentage of cell distribution data for each treatment group shown is the mean of two plates and representative of three independent experiments.

buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 80 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 0.1% Tween 20, 10% glycerol, 1 mM PMSF, and 10 μg/ml leupeptin and aprotonin) and twice with Rb-kinase assay buffer (50 mM HEPES-KOH, pH 7.5, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, 10 mM MgCl₂, 0.1 mM sodium orthovanadate and 1 mM DTT). Phosphorylation of Rb was measured by incubating the beads with 30 μl of ‘hot’ Rb-kinase solution (2 μg of Rb-GST fusion protein, 5 μCi of [γ-32P]ATP, 0.1 mM ATP in Rb-kinase buffer) for 30 min at 37°C. Reaction was stopped by boiling the samples in 5X SDS sample buffer for 5 min. Samples were analyzed by SDS–PAGE, and the gel was dried and subjected to autoradiography. Non-specific anti-serum control was also used to monitor if there were any non-specific immunoprecipitation in all these studies.

Apoptotic cell death assay

IP6 caused cell death at higher doses and longer treatment times and that was analyzed for apoptosis. To quantify IP6-induced apoptotic death of human prostate carcinoma DU145 cells, annexin V and PI staining was performed followed by flow cytometry, as described recently (26). Briefly, after IP6 treatment (1, 2 and 4 mM IP6 for 48 and 72 h), both floating and attached cells were collected and subjected to annexin V and PI staining using Vybrant Apoptosis Assay Kit2 (Molecular Probes, Eugene, OR) and following the step-by-step protocol provided by the manufacture. The kit contains recombinant annexin V conjugated to fluorophores and the Alexa fluoro 488 dye, providing maximum sensitivity. In apoptotic cells, annexin V binds to phosphatidyserine, which is translocated from inner to outer leafllet of the plasma membrane. The apoptotic cells stained with annexin V showed green fluorescence, dead cells stained with both annexin V and PI showed red and green fluorescence, and live cells showed little or no fluorescence.

Statistical and densitometric analyses

The data were analyzed using the Jandel Scientific SigmaStat 2.0 software. For all measurements, one way ANOVA followed by Student’s t-test was employed to assess the statistical significance of difference between control and IP6-treated groups. A statistically significant difference was considered to be present at P < 0.05. Autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA), and adjusted for brightness and contrast for minimum background. The mean density of each band was analyzed by the Scanimage program (NIH, Bethesda, MD). Densitometric data presented below bands are fold change compared with control for each treatment time.

Results

IP6 inhibits growth of human prostate carcinoma DU145 cells

As shown in Figure 1, IP6 treatment inhibited the growth of human prostate carcinoma DU145 cells in a dose- and time-dependent manner. IP6 treatment at 0.25, 0.5, 1 and 2 mM doses for 24–72 h resulted in 13–22 (P < 0.05), 22–29 (P < 0.05), 31–40 (P < 0.01–0.001) and 35–54 (P < 0.01–0.001) % inhibition of cell growth, respectively (Figure 1). Much stronger cell growth inhibition (54–77%, P < 0.001) was observed at 4 mM dose of IP6 treatment for 24–72 h (Figure 1), and virtually the cell number did not increase after 48 and 72 h following addition of treatment media to culture plates. We reasoned that apoptosis might also be a contributor in IP6-caused growth inhibition of DU145 cells, which was investigated later.

IP6 induces G1 arrest in DU145 cells

G1 arrest can prevent the replication of damaged DNA and, therefore, is helpful in checking the uncontrolled proliferation of cancer cells. Based on the growth inhibitory response of IP6 in DU145 cells, we next examined its effect on cell cycle progression. Consistent with its effect on cell growth inhibition, IP6 induced significant (P < 0.05–0.001) G1 arrest in DU145 cells (Figure 2). IP6 treatment (0.25–4 mM IP6) for 24 h resulted in accumulation of 51–60% cells in G1 phase compared with control showing 38%. The observed increase in G1 cell population was accompanied by a decrease in the number of cells in both S phase as well as G2-M phase. In time kinetics,
induced the protein levels of Cip1/p21 and Kip1/p27 (Figure 3) (0.5, 1 and 2 mM IP6 for 24 and 48 h) of DU145 cells strongly with G1 arrest. Western blot analysis showed that IP6 treatment enhanced levels of which have been shown to be associated with G1 arrest in cell cycle progression. Since DU145 cells do not have functional p53 protein, our focus was to assess the protein expression of CDKIs, the tumor suppressor protein associated with the G0-G1 arrest in cell cycle progression. p53 is the most important tumor suppressor gene that is closely associated with the induction of G1 arrest that was investigated in subsequent studies.

**Effect of IP6 on G1 cell cycle regulator**

Based on the data showing that IP6 induces strong G1 arrest in DU145 cells, we assessed the effect of IP6 on the cell cycle regulatory molecules that play important roles in G1 phase of cell cycle progression. p53 is the most important tumor suppressor protein associated with the G0-G1 arrest in cell cycle. Since DU145 cells do not have functional p53 protein, our focus was to assess the protein expression of CDKIs, the enhanced levels of which have been shown to be associated with G1 arrest. Western blot analysis showed that IP6 treatment (0.5, 1 and 2 mM IP6 for 24 and 48 h) of DU145 cells strongly induced the protein levels of Cip1/p21 and Kip1/p27 (Figure 3) in a dose-dependent manner. In the time kinetics, maximum inducible effect of IP6 on CDKIs was evident at 24 h and that was also positively correlated to its effect on G1 arrest. Compared with controls, at the investigated doses and time points, 2 mM IP6 caused a maximum induction in the protein levels of both Cip1/p21 (by 2.5-fold) and Kip1/p27 (by 9-fold) after 24 h of treatment (Figure 3). The observed strong induction in Cip1/p21 and Kip1/p27 protein levels by IP6 was not due to an overall change in protein levels as confirmed by probing the same membranes with actin antibody (Figure 3).

Perturbations in cell cycle regulation have been demonstrated as one of the most common features in cancer cells. These alterations are generally associated with uncontrolled cell growth involving a lack of CDKI or loss of its function and increased expression of CDKs and cyclins (27). Accordingly, we next assessed the effect of IP6 on the protein levels of CDKs and cyclins involved in G1 phase and G1 to S phase transition of cell cycle progression. As shown in Figure 3, IP6 treatment of DU145 cells (0.5, 1 and 2 mM IP6 for 24 and 48 h) did not show any noticeable change in the protein levels of CDK2, 4 and 6, and cyclin D1, D3, E and A except a slight increase in cyclin D2 after 24 h of treatment (Figure 3).

**IP6 increases cyclin-CDKI binding and decreases kinase activities associated with CDK2, 4 and 6, and cyclin D1 and E**

Since up-regulation in CDKI level was observed maximally at 2 mM IP6 treatment for 24 h, this was selected for CDK-CDKI binding and kinase studies. As shown in Figure 4A–C, IP6 showed an increased binding Cip1/p21 and Kip1/p27 with cyclin D1 and E. The bound level of Kip1/p27 with these cyclins was strongly increased following IP6 treatment that was correlated with increased expression of these CDKIs. Densitometric analysis (Figure 4B,C) showed 1.4-fold increase in the bound protein level of Cip1/p21 whereas in the case of Kip1/p27 the increase was 6–10-fold compared with control. Since increased binding of CDKIs to CDK-cyclin complex subsequently decreases kinase activity of CDK-cyclin complex, we next investigated the effect of IP6 on CDK2, 4 and 6, and cyclin D1 and E associated kinase activity. As shown in Figure 4D, IP6 treatment of DU145 cells at 2 mM dose for 24 h strongly decreased the histone H1-associated kinase activities of CDK2 and cyclin E immunoprecipitates. Similarly, IP6 also decreased Rb-associated kinase activities of CDK4, and 6, and cyclin D1 immunoprecipitates (Figure 4D). In all these studies, specificity of immunoprecipitation and kinase assays was compared with antiserum control that did not show any such specific bands observed in assays (data not shown). Densitometric analysis showed that kinase activities were inhibited by 50–80%, which might be due to up-regulation of CDKIs and their increased binding with CDK-cyclin complexes. Together, these results suggest that growth inhibitory effect of IP6 in DU145 cells could be via an induction of CDKIs leading to a decrease in kinase activity of CDKs followed by modulation of further down-stream targets such as pRb-related proteins and E2F family of transcription factors. This suggestion, therefore, was next explored.

**IP6 increases hypophosphorylated levels of pRb/p107 and pRb2/p130, and their binding with E2F4**

One of the down-stream targets of the cyclin kinases is the pRb-related proteins in the cells lacking functional pRb protein (28). Since IP6 showed a strong decrease in kinase activity associated with CDK2, 4 and 6, and cyclin D1 and E, our next focus was to investigate the phosphorylation status of...
IP6 induces G1 arrest and apoptosis in PCA cells

Fig. 4. Effect of IP6 on cyclin-CDKI complex, and CDK and cyclin-associated kinase activities. Cells were cultured in RPMI 1640 medium with 10% FBS, and treated with either vehicle or 2 mM doses of IP6 for 24 h as described in ‘Materials and methods’. At the end of treatments, total cell lysates were prepared. (A) In binding study, cyclin D1 and E were immunoprecipitated using specific antibody from 200 mg of total protein lysates followed by SDS-PAGE and western immunoblotting. Membranes were probed for Cip1/p21, Kip1/p27, cyclin D1 and E. (B and C) Densitometric data of bound levels of Cip1/p21 and Kip1/p27 with cyclin D1 and E, presented as fold change of control after IP correction. (D) Effect of IP6 on CDK2, 4 and 6, and cyclin D1 and E-associated kinase activities. Treatments and cell culture conditions were the same as those in binding studies. CDK2 and cyclin E kinase activities were determined by in-bead histone H1 kinase assay using immunoprecipitated CDK2 and cyclin E from total cell lysates (200 µg protein) using specific antibody; and CDK4, 6 and cyclin D1-associated kinase activities were determined by in-bead RB–GST fusion protein kinase assay using immunoprecipitated CDK4, 6 and cyclin D1 from total cell lysates (200 µg protein) using specific antibodies as described in ‘Materials and methods’. After the assay, the labelled substrates were subjected to SDS–PAGE, and the gel was dried and exposed to X-ray film. The bottom panel of each band indicates the fold change in band intensity compared with that of control at each treatment time. The experiments were repeated 2–3 times with similar results. IB, western immunoblotting; IP, immunoprecipitation.
pRb-related proteins pRb/p107 and pRb2/p130. IP6 treatment of DU145 cells at 0.5–2.0 mM doses for 24 h, showed increase in hypophosphorylated levels of pRb/p107 and pRb2/p130 in a dose-dependent manner (Figure 5A). Next, we examined the protein levels of E2F3, 4 and 5, as E2Fs are the target transcription factors in this pathway that regulate the transcription of growth responsive genes. Similar IP6 treatment of DU145 cells did not show any observable change in E2F3 and 5 protein levels (data not shown), but moderately decreased the level of E2F4 (Figure 5A). Protein loading was checked by re-probing of the same membrane with actin antibody (Figure 5A). Since, oncogenic activity of E2Fs is dependent on their free/bound ratio with pRb-related proteins in pRb lacking cells, next we determined the bound levels of E2F3, 4, and 5 with pRb/p107 and pRb2/p130 after IP6 treatment. As shown in Figure 5B, IP6 showed an increased binding of E2F4 with pRb/p107 and pRb2/p130, which accounted for almost 2–2.5 fold increase as compared with that of control (Figure 5C). There was no noticeable change in the bound levels of E2F3 and 5 with pRb/p107 and pRb2/p130 (data not shown). The bound level of E2F4 with pRb2/p130 was more prominent compared with pRb/p107. Since pRb2/p130-E2F4 has been reported as the most abundant complex in the resting or quiescent cells in G0 (29), the observed effect of IP6 on an induction of this complex level could be of greater significance in defining a mechanistic rationale involved in IP6-caused G1 arrest and cell growth inhibition, and possibly also in apoptotic death which was investigated next.

**IP6 causes apoptotic death of DU145 cells**

We investigated any possible involvement of apoptosis induction by IP6 that might have contributed in its strong growth
inhibitory effect at higher doses and longer treatment times. A quantitative apoptotic cell death study was performed to answer this question. IP6-caused apoptotic death of DU145 cells was analyzed by annexin V/PI staining which showed that higher dose (4 mM) treatment of IP6 for 48–72 h significantly (P < 0.05) increased the percentage of apoptotic cells up to 3–4 fold accounting for 9–14% compared with that of control showing 3–4% (Figure 6A). During same treatment time the lower doses (1 and 2 mM) of IP6 could maximally induce up to 1.5 fold apoptotic cell death (Figure 6A). To further confirm the apoptotic effect of IP6, western blot analysis was performed to analyze PARP cleavage and caspase 3 (active fragment). As shown in Figure 6B, IP6 increased the levels of both cleaved PARP and caspase 3, and therefore suggested the possible involvement of caspasases activation as one of the possible mechanisms of apoptosis induction, which is yet to be investigated.

Discussion

The major finding of the present study is that IP6 strongly induces Cip1/p21 and Kip1/p27 and inhibits CDK2, 4, and 6, and cyclin D1 and E-associated kinase activities followed by an increase in hypophosphorylated levels of pRb/p107 and pRb2/p130, and their increased binding with E2F4. These molecular effects of IP6 could be one of the possible underlying mechanisms that resulted in inhibition of cell growth and G1 arrest in DU145 cell cycle progression. Furthermore, IP6 causes apoptotic cell death in human prostate carcinoma cells that was evidenced by the cleavage of PARP and caspase 3.

The results obtained in the present study provide convincing evidence that IP6 exerts its effects on cell cycle progression mainly via an up-regulation of Cip1/p21 and Kip1/p27. When cell cycle phase distributions are compared with alterations in cell cycle regulatory molecules, a strong increase in CDKIs can be attributed as one of the major causes of IP6-induced G1 arrest and cell growth inhibition. This finding is consistent with earlier reports in which IP6 has been shown to induce Cip1/p21 and G1 arrest in breast cancer cells (30,31). However, it should also be noted that IP6 induces G2-M arrest in leukemia cell lines by down-regulation of CSK2 (cdc 28 protein kinase 2) and Kip2/p57, suggesting a different mechanism of cell cycle arrest by IP6 and that could be most likely due to dissimilar origin of these cells (32). The mammalian cell cycle is regulated by complex machinery, in which CDKs, CDKIs and cyclin play essential roles (27). CDKIs are tumor suppressor proteins that down regulate the cell cycle progression by binding with active CDK-cyclin complexes and thereby inhibiting their kinase activities (33,34). The important CDKIs include Cip1/p21, a universal inhibitor of CDKs whose expression is mainly regulated by the p53 tumor suppressor protein (35); and Kip1/p27 that is also up regulated in response to antiproliferative signals (36). The increased expression of G1 cyclins in cancer cells provide them an uncontrolled growth advantage because most of these cells either lack CDK1 or possess non-functional CDK1 or have low expression of CDK1 (37). The increased expression of CDKIs by IP6 is both encouraging and important in the sense that decreased Kip1/p27 expression in prostatic carcinomas has been associated with aggressive phenotype, and loss of Cip1/p21 function has been implicated in the failure of irradiation response (38). Additionally, our data suggest that IP6-caused CDKIs up-regulation involves p53-independent pathway, as DU145 cells lack functional p53.

The pRb-related proteins, pRb/p107 and pRb2/p130 cooperate to regulate cell cycle progression through G1 phase of cell cycle with a common goal of G1-S checkpoint regulation. It has been reported that pRb2/p130 and Kip1/p27 are mutually involved in a negative regulation of cellular proliferation (24). The Rb family members are nuclear phospho-proteins and are regulated in a cell cycle-dependent manner by phosphorylation, and are critical targets for inactivation by transforming oncogenes (24,28). pRb/p107 and pRb2/p130 show considerable homology with pRb and classified as a sub-family of pRb family members. These proteins bind to and modulate the activity of the E2F family of transcription factors that induce the transcription of genes needed for the cell cycle progression through S phase (28). The pRb2/p130-E2F4 is the most abundant E2F complex found in a resting or quiescent cells in G0, and is suggested to help in maintaining a state of transcriptional silence (29). Consistent with these reports, IP6 caused a decrease in CDK-cyclin kinase activity, and increased hypophosphorylated levels of Rb-related proteins and their increased binding with E2Fs may be attributed as decreased expression of growth responsive genes and subsequent growth inhibition of PCA cells by IP6. E2Fs have been identified as down-stream targets for the action of the tumor suppressor proteins p53 and pRb (39). An increase in E2F transcriptional activity as a consequence of loss of p53 and pRb, that is the case in DU145 cells, is considered to be a critical factor in deregulated cell cycle progression, a common phenomenon in tumorigenesis (29). The Rb-related proteins and associated factors are suggested to be critical targets in cancer therapy. Accordingly, the observed biological effects of IP6 in DU145 cells and their associated mechanisms are encouraging, and need more detailed study to justify the modulation of these molecular events towards anticancer effect of IP6 against human PCA.

It is well established that apoptosis and, the associated signalling pathways and cellular events controlling it have a profound effect on the progression of benign to malignant phenotype and can be targeted for the therapy of various malignancies including PCA (40). Accordingly, our results showing induction of apoptotic death of DU145 cells by IP6 could be of greater significance in identifying another (together with growth inhibitory) anticancer effect of IP6 in PCA. Since induction of CDKIs has been reported in anticancer agent-induced apoptosis in human PCA cells (41), the IP6-caused increase in CDKIs could be in part responsible for the observed apoptotic death of DU145 cells. PARP cleavage and increase in the level of active caspase 3 suggested that caspases activation could have an important role in IP6-induced apoptosis induction. However, the detailed studies are needed to describe IP6-induced apoptotic death of DU145 cells.

The IP6 doses (0.25–4 mM) used in the present study were based on earlier published studies, which in most cases used up to 5 mM IP6 concentration in cell culture treatments (16–18,30,31). The physiological and/or pharmacological significance of these levels of IP6 in cell culture studies needs to be established in future in both animal studies as well as in humans. In this regard, it is important to re-emphasize that endogenous mammalian cellular concentration of IP6 ranges from 0.01–1 mM (7). However, most effects reported in the present study are at 2 mM dose in cell cycle studies and are at 4 mM dose in both cell cycle and apoptosis studies. Similarly, other biological effects of IP6 reported in literature are also at 2–5 mM doses in cell culture (16–18,30,31).
Comparing these concentrations, it is obvious that the endogenous cellular/physiological level of IP6 is not sufficient for its anti-cancer activity at least in cell culture, and that higher levels of IP6 are needed for its pharmacological efficacy. When these cell culture concentrations of IP6 used in the present study and in earlier reports (with only one treatment) were compared with published literature showing its anti-cancer activity in animal models by using up to 15 mM dose in continuous feeding in drinking water (reviewed in ref. 10), concentrations in cell culture studies are much lower, which clearly establish their relevance in defining molecular mechanism of efficacy of IP6 at pharmacological concentrations related to pre-clinical studies. The clinical efficacy of IP6 against any cancer is an open area, and has to be investigated and established in future. However, a recent study on oral absorption of IP6 in humans has reported that IP6 concentration in plasma could go up by 3–5-fold (~0.3 mg/l) by ingestion of IP6-normal diet when compared with the ingestion of IP6-poor diet (42). This study also reported that humans become deficient in IP6 if they consume IP6-poor diet for as little as 2 weeks, and that the normal level of IP6 could be easily achieved by IP6 dietary supplement. These findings suggest that a dose-escalation study with IP6 is needed to assess the pharmacologically achievable concentrations of IP6 as well as associated toxicity, if any.

Several studies suggest that androgen-independent prostate tumors contain an altered form of androgen receptor, evolving from gene rearrangement, point mutation or deletion, leading to the presence of a receptor that remains (constitutively) active even in the absence of androgen or presence of anti-androgen (43). This hormone refractory PCA phenotype is associated with several changes at molecular level including mutations of androgen receptor protein, alteration in mitogenic and cell survival signalling, inactivation of p53 and loss of pRb (44). There is no effective standard chemotherapy for the patients diagnosed with hormone-refractory PCA, which often results from androgen ablation therapy that invariably fails to prevent the progressive metastatic disease. In such patients, median survival time is only 6–9 months (45). One possible practical and translational approach in controlling the growth and metastatic potential of PCA in those patients as well as increasing the survival time and quality of life could be through dietary agents. In this regard, IP6 has already shown promising results in several animal tumor studies as well as epidemiological reports related to mammary, colon and prostate cancers. Based on these reports, recent studies by us, and the data of the present work, a recommendation could be made that further studies are needed to establish the relevance of observed in vitro anticancer effects of IP6 under in vivo pre-clinical prostate cancer models, and may be helpful in developing IP6 as an effective cancer preventive agent against advanced human PCA.

Acknowledgement
This work was supported by USPHS grant CA83741 from the National Cancer Institute, NIH.

References
IP6 induces G1 arrest and apoptosis in PCA cells


Received October 24, 2002; revised December 2, 2002; accepted December 10, 2002