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Research Article

p21/Cip1 and p27/Kip1 Are Essential Molecular Targets of Inositol Hexaphosphate for Its Antitumor Efficacy against Prostate Cancer

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Abstract

Inositol hexaphosphate (IP6) causes G_1 arrest and increases cyclin-dependent kinase inhibitors p21/Cip1 and p27/Kip1 protein levels in human prostate cancer (PCa) DU145 cells lacking functional p53. However, whether cyclin-dependent kinase inhibitor I induction by IP6 plays any role in its antitumor efficacy is unknown. Herein, we observed that either p21 or p27 knockdown by small interfering RNA has no considerable effect on IP6-induced G_1 arrest, growth inhibition, and death in DU145 cells; however, the simultaneous knockdown of both p21 and p27 reversed the effects of IP6. To further confirm these findings both *in vitro* and *in vivo*, we generated DU145 cell variants with knockdown levels of p21 (DU-p21), p27 (DU-p27), or both (DU-p21+p27) via retroviral transduction of respective short hairpin RNAs. Knocking down p21 or p27 individually did not alter IP6-caused cell growth inhibition and G_1 arrest; however, their simultaneous ablation completely reversed the effects of IP6. In tumor xenograft studies, IP6 (2% w/v, in drinking water) caused a comparable reduction in tumor volume (40–46%) and tumor cell proliferation (26–28%) in DU-EV (control), DU-p21, and DU-p27 tumors but lost most of its effect in DU-p21+p27 tumors. IP6-caused apoptosis also occurred in a Cip/Kip-dependent manner because DU-p21+p27 cells were completely resistant to IP6-induced apoptosis both in cell culture and xenograft. Together, these results provide evidence, for the first time, of the critical role of p21 and p27 in mediating the anticancer efficacy of IP6, and suggest their redundant role in the antiproliferative and proapoptotic effects of IP6 in p53-lacking human PCa cells, both *in vitro* and *in vivo*. [Cancer Res 2009;69(3):1166–73]

Introduction

Prostate cancer (PCa) accounts for ~25% (186,320) of cancer incidence and is the second leading cause of cancer-related deaths in the male population in the United States (1). The disease is initially responsive to androgen ablation therapy, but it eventually progresses to the androgen-independent form which is relatively more invasive and metastatic (2). Usually, PCa is a disease of elderly males and the time elapsed between the onset of preneoplastic lesions and detectable malignancy often ranges in decades, thus allowing for a sufficiently large time window for preventive intervention. However, the use of synthetic agents in long-term chemopreventive strategies is intrinsically associated with safety and toxicity problems (3, 4). This has led to the increasing enthusiasm in diet-related cancer chemoprevention strategies, which are also supported by epidemiologic and case-control studies indicating an important role of dietary agents in the etiology as well as the prevention of cancer (5–7). Low-fat and high-fiber (especially soluble fibers present in oat bran, legumes, etc.) diets have been suggested to protect against various forms of cancers including PCa (8).

Inositol hexaphosphate (IP6) is a polyphosphorylated carbohydrate present in abundant amounts in high-fiber–containing food sources such as legumes, nuts, cereals, etc. IP6 and its lower phosphate forms (IP1–IP5) are also present in mammalian cells, in which they govern various cellular functions, including signal transduction, growth, and differentiation (9). IP6 is available as a dietary supplement and has long been known for its beneficial effects, including strengthening the immune system, preventing kidney stone formation and lowering serum cholesterol levels (9–11). Shamsuddin and coworkers first established the cancer-preventive efficacy of IP6 in carcinogen-induced colon cancer models (12), following which there has been an increasing interest in investigating the antineoplastic potential of this phytonutrient. IP6 has shown significant promise against a broad spectrum of cancer models including prostate, colon, pancreas, liver, and breast (13–18). Moreover, in breast cancer models, when used in a combination regimen with Adriamycin or tamoxifen, IP6 increases the activity of these conventional chemotherapeutic agents and helps to overcome drug resistance (19). Over the past few years, our laboratory has focused on establishing the chemopreventive efficacy of IP6 in cell culture and animal models of PCa. Initial studies showed that IP6 induces prominent G_1 arrest as well as apoptosis in both androgen-dependent and androgen-independent PCa cell lines (20, 21). In tumor xenografts of DU145 cells, IP6 significantly reduced tumor volume, tumor weight, cell proliferation and angiogenesis, and induced apoptosis (22). Furthermore, in the transgenic adenocarcinoma of mouse prostate model, which is a reliable murine model for studying the multistage events of prostate carcinogenesis, IP6 treatment showed a significant reduction in tumor grade and cell proliferation, and an induction of apoptosis in prostate tumor tissues (23). There is no report, thus far, of any considerable toxicity of IP6 in animal studies (16, 17, 22, 23). The promising anticancer efficacy, along with the lack of toxicity associated with IP6 administration in preclinical models, underscores the chemopreventive potential of IP6 as well as warranting further investigation of its molecular mechanism of action.

Threshold kinase activity of cyclin-dependent kinases (CDK) is a crucial determinant of the cell cycle progression, and thus, the

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agents which inhibit CDK activity directly or indirectly, by up-regulating CDK inhibitor (CDKI) expression, represent a rational approach to intervene with the uncontrolled proliferation of carcinoma cells (24). Moreover, prolonged cell cycle arrest resulting in apoptosis is also an effective strategy in reducing the burden of cancer cells. The key features of IP6 efficacy, as established in various PCa cell lines, are cell cycle arrest, apoptosis, and concurrent Cip/Kip protein induction (20, 21). In order to address the question of whether the biological effects of IP6 are mediated in a Cip/Kip-dependent manner, we generated DU145 cells with stable knockdown levels of p21 and/or p27 proteins and examined the effect of IP6 on these cell variants for cell proliferation, growth arrest, apoptosis, and tumorigenicity. Our studies reveal that induction of p21 and p27 protein expression is an essential event for mediating the antitumor, antiproliferative, and apoptotic effects of IP6.

Materials and Methods

Cell lines and small interfering RNA transfection. p27 small interfering RNA (siRNA; sequence not revealed by vendor) was from Upstate, p21-siRNA (sequence not revealed by vendor) was from Cell Signaling Technology, nonspecific (control)-siRNA was from Dharmacon, and Trans-IT TKO transfection reagent was from Mirus. DU145 cells (American Type Culture Collection) were grown in RPMI 1640 with 10% serum, and at ~30% confluency, transfected with nonspecific, p21, and/or p27 siRNA (each 50 nmol/L, final concentration) in 10% serum condition for 24 h following Mirus's protocol with some modifications. The next day, cells were fed with fresh medium with or without 2 mmol/L of IP6 for 24 h, and harvested for cell cycle analysis or Western immunoblotting. The selection of 2 mmol/L of IP6 concentration for all the cell culture studies was based on our recent publications showing optimum effects at this level (17, 20, 21). DU145 cell variants with empty vector (DU-EV) and knockdown levels of p21 (DU-p21), p27 (DU-p27), or both (DU-p21+p27) were generated via retroviral transduction of respective short hairpin RNAs as reported recently (25).

Cell growth and clonogenic assay. For growth curves, 1×10^5 DU145 cell variants were plated in 60 mm dishes, treated with 2 mmol/L of IP6 for 48 h, and then the number of cells and percentage of dead cells were analyzed by trypan blue exclusion method as reported recently (20, 21). To assess the clonogenic potential of these different DU145 cell line variants and IP6 activity, cells were seeded at a density of 1,000 cells/well in six-well plates and next day were treated with 2 mmol/L of IP6. After 10 days, cells were fixed in methanol/acetic acid (3:1) for 10 min and then stained with 0.1% crystal violet for 30 min. The plates were washed thrice with PBS and the colonies (>50 cells/colony) were counted under Zeiss Invertoskop 40C (Carl Zeiss, Inc.).

Flow cytometry analysis for cell cycle distribution. Following the desired treatments, cells were harvested and quickly washed twice with ice-cold PBS, and cell pellets were collected. Approximately 0.5×10^6 cells in

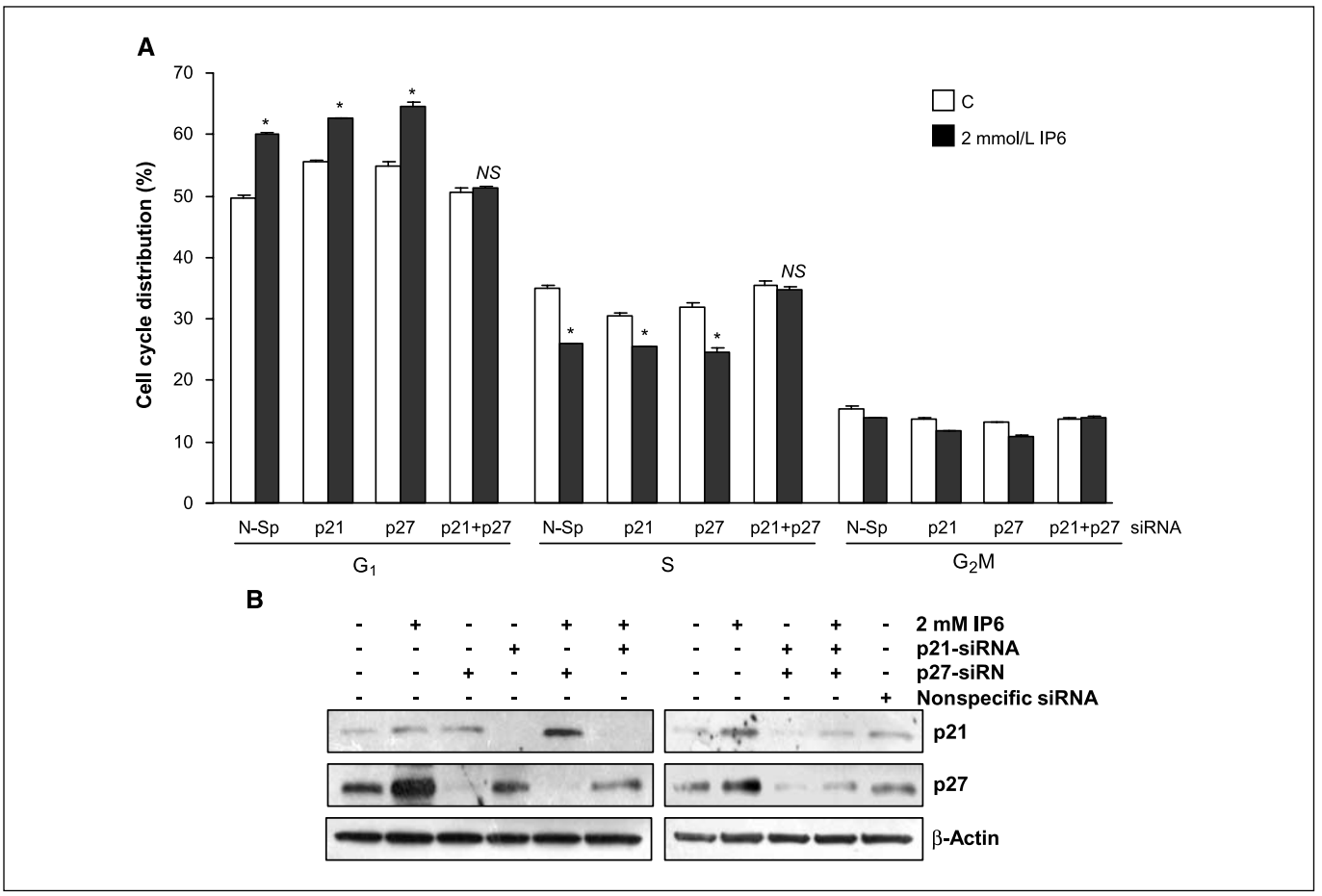

Figure 1. IP6 fails to induce prominent G_1 arrest in cells transiently transfected with both p21 and p27 siRNAs. DU145 cells were transfected with siRNAs for p21 and/or p27, and then treated with or without 2 mmol/L of IP6 for 24 h as detailed in Materials and Methods. Cells were harvested and analyzed for cell cycle distribution (*A*), or cell lysates were prepared and immunoblotting was performed for p21, p27, and β-actin protein levels (*B*) as detailed in Materials and Methods. *C,* control; *N-Sp,* nonspecific; *, $P < 0.001$; *NS,* not statistically significant.

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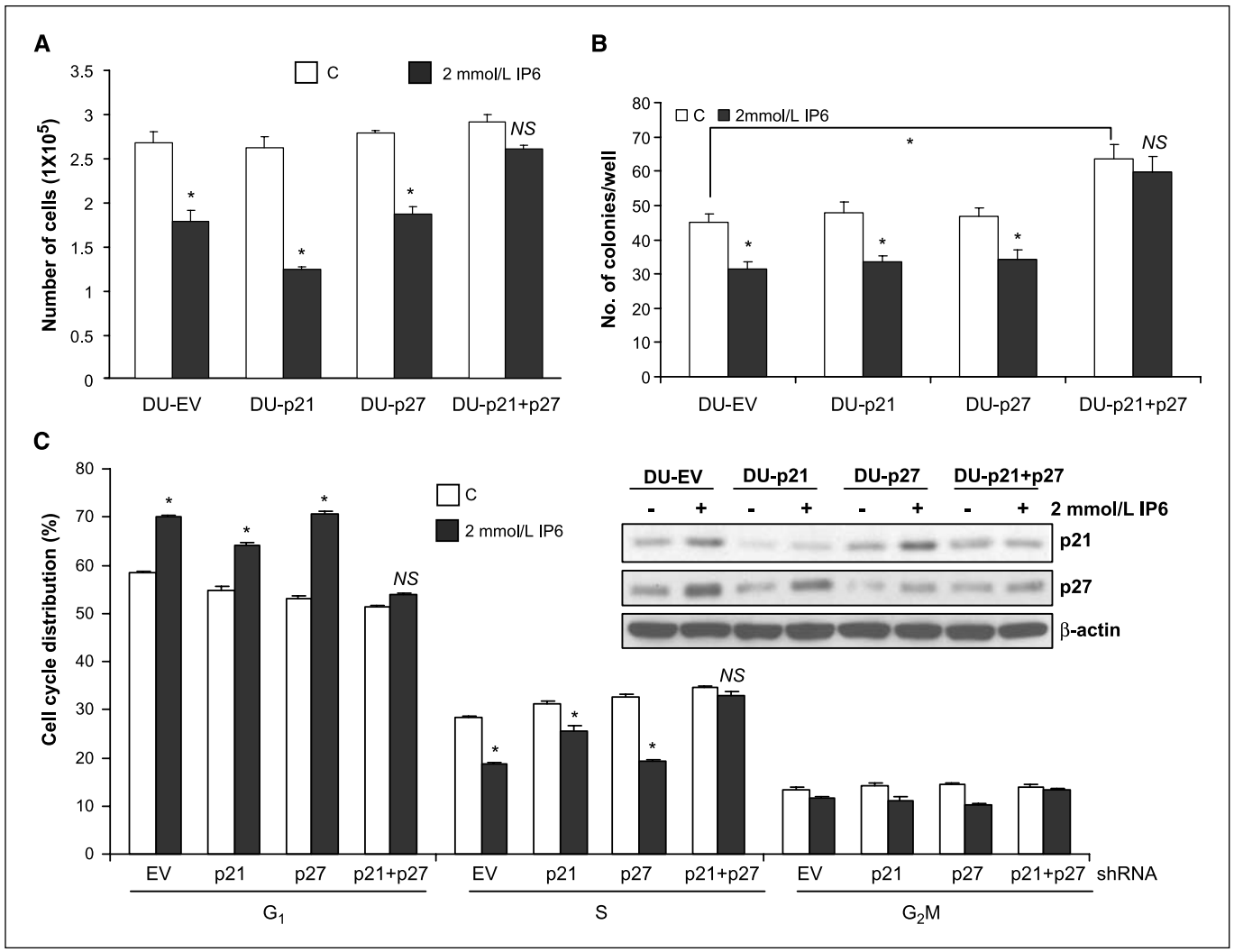

Figure 2. Knocking down p21 and p27 abrogates the growth arrest response of IP6 in DU145 cells. DU145 cell variants were treated with or without 2 mmol/L of IP6 for 48 h and the number of viable cells (*A*) was determined as detailed in Materials and Methods. For clonogenic potential (*B*), DU145 cell line variants were seeded at a density of 1,000 cells/well in six-well plates and the next day treated with or without 2 mmol/L of IP6. After 10 days, cells were fixed in methanol/acetic acid, stained with crystal violet, and the colonies were counted under an inverted microscope as detailed in Materials and Methods. *C,* DU145 cell variants were treated with or without 2 mmol/L of IP6 for 48 h and then analyzed for cell cycle distribution, or cell lysates were prepared and immunoblotting was performed for p21, p27, and β-actin protein levels (*inset*) as detailed in Materials and Methods. *C,* control; *, $P < 0.001$; *NS,* not statistically significant.

0.5 mL of saponin/propidium iodide (PI) solution (0.3% saponin, w/v; 25 μg/mL PI, 0.1 mmol/L EDTA; and 10 μg/mL RNase A) were incubated at 4°C for 24 h in the dark as reported earlier (26). Cell cycle distribution was then analyzed by flow cytometry using the FACS Analysis Core Services of the University of Colorado Cancer Center. The quantitative data presented are mean ± SE of the percentage cell population in different phases of cell cycle from triplicate samples in each treatment, and were reproducible in two independent experiments.

Western immunoblotting. Total lysates, from cells in culture and tumors in xenograft studies, were prepared in nondenaturing lysis buffer as reported previously, and subjected to SDS-PAGE on 12% Tris-glycine gel (26). The separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% nonfat milk powder (w/v) in TBS [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 0.1% Tween 20] for 1 h at room temperature. Membranes were probed with antibodies for p21 and p27 (Millipore), and cleaved PARP (Cell Signaling Technology) followed by peroxidase-conjugated appropriate secondary antibody and visualized by enhanced chemiluminescence detection system (GE Healthcare Bioscience). In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. The blots were scanned with Adobe Photoshop 6.0 (Adobe Systems), and the mean density of each band was analyzed by the Scion Image program (NIH, Bethesda, MD). As needed, the densitometric values are given below each band. To ensure equal protein loading, each membrane was stripped and reprobed with anti–β-actin antibody (Sigma).

***In vivo* tumor xenograft study.** Cells were detached from the culture dishes by trypsinization and then collected, washed, and resuspended in serum-free and antibiotic-free RPMI 1640. To establish DU145 cell variants, tumor xenografts in mice, 6-week-old athymic *nu/nu* male mice were injected s.c. with 3×10^6 cells mixed with Matrigel (1:1; Collaborative Biomedical Products) in the right flank of each mouse. The animals of each cell type were then divided into two groups ($n = 14$ mice per group) and from day 6 were fed with regular drinking water or with 2% IP6 (w/v) in drinking water. The tumor sizes were measured twice weekly with a digital caliper, and tumor volume was calculated by the formula "$0.5236\ L_1\ (L_2)^2$, where L_1 is the long axis and L_2 is the short axis of the tumor" as reported previously (27). At the end of the experiment, tumors were excised, weighed, and stored at −80°C until additional analysis.

Immunohistochemistry. Part of tumor samples were fixed in 10% buffered formalin for 12 h and processed conventionally. For proliferating

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cell nuclear antigen (PCNA) staining, the paraffin-embedded tumor sections (5 μm thick) were heat-immobilized, deparaffinized using xylene, and then rehydrated in a graded series of ethanol followed by antigen retrieval and blocking of endogenous peroxidase activity as reported (28). Sections were then incubated with anti-PCNA antibody (Dako), and then incubated with biotinylated secondary antibody, streptavidin and 3,3′-diaminobenzidine (Sigma). Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediating dUTP nick end labeling (TUNEL) staining using Dead End Colorimetric TUNEL System (Promega) according to the manufacturer's protocol. For quantification, PCNA- or TUNEL-stained cells were counted in five arbitrarily selected fields at 400× magnification and data are represented as the number of positive (brown) cells × 100 / total number of cells (28).

Statistical and immunohistochemical analyses. Statistical significance of difference between the control and treated group of each cell type as well as between different cell types was determined by one-way ANOVA followed by Bonferroni *t* test using SigmaStat 2.03 software (Jandel Scientific). $P < 0.05$ was considered statistically significant. Microscopic immunohistochemical analysis was done by Zeiss Axioscop 2 microscope (Carl Zeiss Inc.) and photomicrographs were captured by Carl Zeiss AxioCam MrC5 camera.

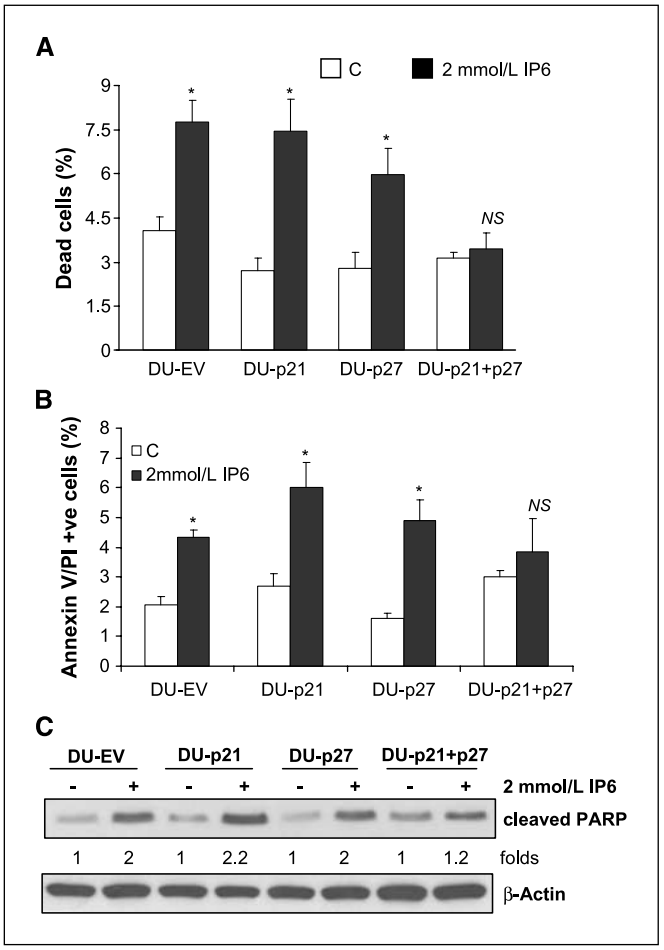

Figure 3. Knocking down p21 and p27 levels makes DU145 cells resistant to IP6-induced cell death and apoptosis. DU145 cell variants were treated with or without 2 mmol/L of IP6 for 48 h, and the percentage of dead cells was analyzed by trypan blue exclusion method (*A*). Under identical treatments, cells were next analyzed for Annexin V/PI staining (*B*), or cell lysates were prepared and immunoblotting was performed for cleaved PARP and β-actin protein levels (*C*) as detailed in Materials and Methods. *C,* control; *, $P < 0.001$.

Results

IP6-induced G_1 arrest is mediated via p21 and p27 induction in DU145 cells. Consistent with our earlier studies, IP6 increased the expression levels of both p21 and p27 proteins which were associated with G_1 arrest mostly at the expense of S phase cell population in DU145 cells (Fig. 1*A* and *B*). In the studies determining the role of these CDKIs in IP6-induced G_1 arrest, knocking down either p21 or p27 by siRNA (Fig. 1*A*) did not reverse the IP6-induced G_1 arrest (Fig. 1*A*), but rather seemed to sensitize the cells to slightly increased G_1 arrest following IP6 treatment (Fig. 1*A*). In p27 knockdown condition, a slight increase in p21 protein level was also observed, which became more prominent following IP6 treatment (Fig. 1*B*). When we used both p21 and p27 siRNAs together to create a double knockdown condition, we did not observe any change in cell cycle phase distribution (versus control) even though protein expression of both the molecules was ablated (Fig. 1*A* and *B*). In addition, knocking down of both these CDKIs also completely reversed IP6-induced G_1 arrest in DU145 cells (Fig. 1*A*). Cells transfected with nonspecific control siRNA did not show any considerable effect on cell cycle distribution (Fig. 1*A*) or the protein expression of these CDKIs (Fig. 1*B*). These results suggest that either of the functional Cip and Kip genes is sufficient to mediate IP6-induced G_1 arrest in the advanced stage of PCa in which both p53 and Rb are mutated. Together, these findings also suggested that CDKIs p21 and p27 are the critical targets of IP6 efficacy in controlling deregulated cell cycle progression via G_1 arrest in PCa cells. Several additional studies were next performed to support this suggestion under both *in vitro* and *in vivo* conditions using stable p21 and/or p27 knockdown DU145 cells.

Down-regulation of p21 and p27 protein levels attenuates the growth arrest efficacy of IP6 via abrogation of the G_1 checkpoint. Because studies with p21 and p27 siRNAs provided convincing evidence of the indispensable role of Cip/Kip proteins in IP6-induced G_1 arrest, we used cell lines with stable knockdown levels of p21 (DU-p21) or p27 (DU-p27) or both (DU-p21+p27) proteins, via retroviral transduction of their corresponding short hairpin RNAs, to elucidate the precise role(s) of these molecules in the anticancer efficacy of IP6 (25). First, we examined the effect of IP6 treatment on the growth characteristics of these different cell variants. After 48 h of treatment with a 2 mmol/L dose of IP6, there were 33% ($P < 0.001$), 52% ($P < 0.001$), and 35% ($P < 0.001$) reduction in total cell number in DU-EV, DU-p21, and DU-p27 cells, respectively; whereas in DU-p21+p27 cells, there was only a 10% (nonsignificant) reduction in cell number compared with controls (Fig. 2*A*). The clonogenic potential of these cell variants was also affected differently with IP6 treatment. The clonogenicity of the untreated DU-EV, DU-p21, and DU-p27 cells were comparable, and with IP6, their colony-forming capacity was reduced by 31%, 30%, and 27%, respectively (Fig. 2*B*). However, the clonogenic potential of DU-p21+p27 cells was higher (41%) than the untreated DU-EV cells, and with IP6 treatment, it was reduced by only 6% (nonsignificant versus control; Fig. 2*B*).

The effect of IP6 on the cell cycle progression pattern of different DU145 cell variants was also analyzed. Consistent with the results obtained in DU145 cells, transiently transfected with p21 and/or p27 siRNAs, IP6 induced significant G_1 arrest in DU-EV cells (58% G_1 population in control versus 68% in IP6-treated group; $P < 0.001$) as well as in DU-p21 (54% in control versus 65% in IP6 group; $P < 0.001$) and DU-p27 cells (53% in control versus 69% in IP6 group; $P < 0.001$; Fig. 2*C*). However, in case of DU-p21+p27 cells,

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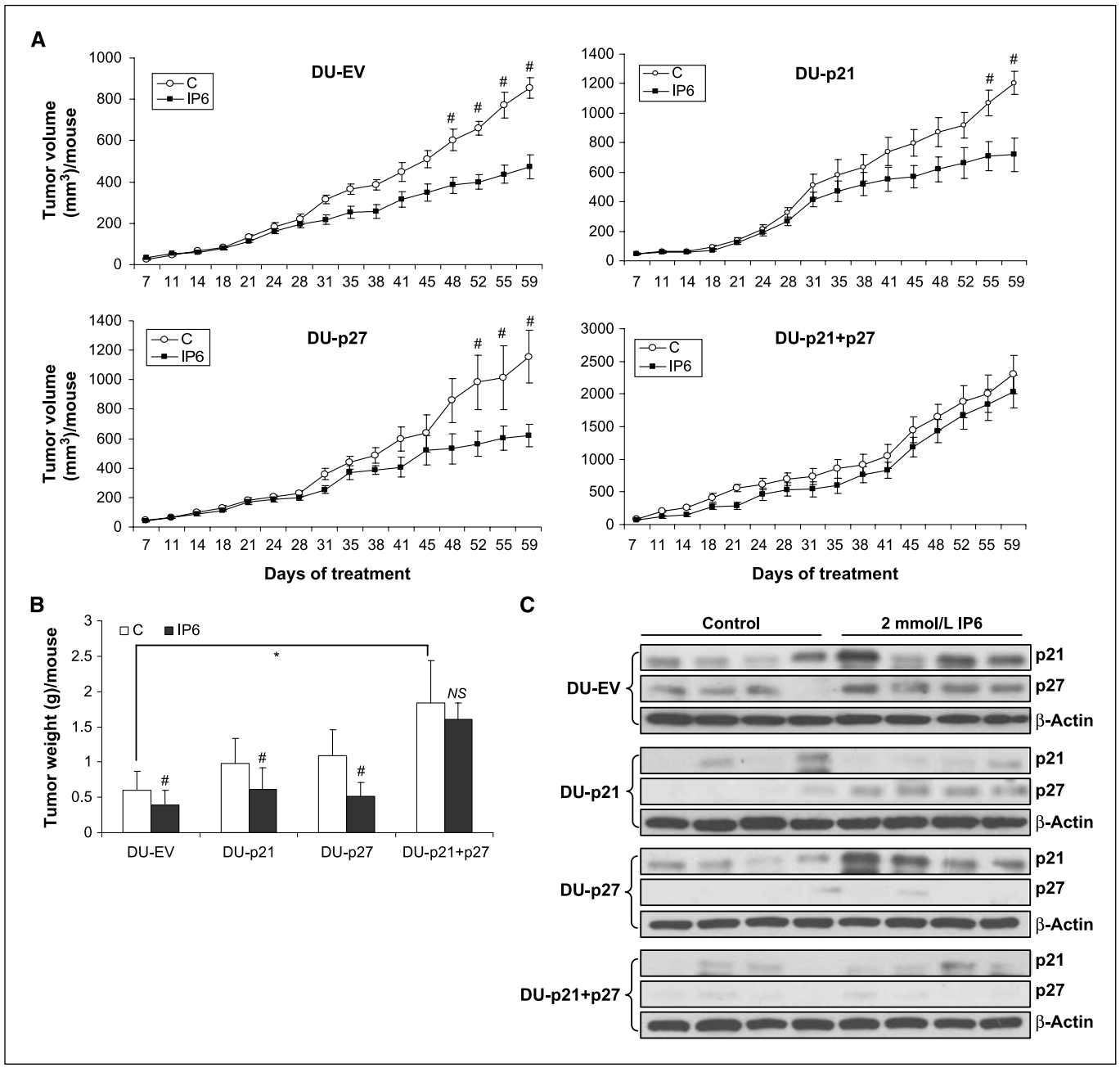

Figure 4. IP6 fails to inhibit tumor xenograft growth of DU145 cells with knocked down levels of both p21 and p27. Different DU145 cell variants were implanted in athymic nude mice as detailed in Materials and Methods. After 6 days of inoculation, animals were fed with or without IP6 (2% w/v) in drinking water. *A,* the tumor volume was measured as a function of days of treatment throughout the study. *B,* at the end of 59 days of treatment, the animals were euthanized and tumor tissues were excised and weighed. *C,* the relative protein levels of p21 and p27 with or without IP6 treatment were analyzed in all the DU145 tumor types by Western blotting. *C,* control; #, $P < 0.05$; *, $P < 0.001$; *NS,* not statistically significant.

the IP6-caused G_1 arrest was almost completely reversed because the G_1 population increased from 51% in control to only 54% with IP6 treatment (Fig. 2*C*). Western immunoblotting for Cip/Kip proteins in these treatment groups showed that IP6 increases both p21 and p27 levels in DU-EV cells, only p27 levels in DU-p21 cells and only p21 levels in DU-p27 cells, but neither of them in DU-p21+p27 cells (Fig. 2*C, inset*). Together, these findings convincingly support the notion that ablation of p21 or p27 protein levels affects the growth-inhibitory potential of IP6 to different degrees; nevertheless, they play complimentary roles for cell cycle arrest and cell growth inhibition, and their simultaneous ablation is essential to reverse the antiproliferative efficacy of IP6.

Essential role of p21 and p27 in apoptotic response of IP6 in DU145 cells. Our previous studies have shown that IP6 induces moderate to high levels of apoptotic death in DU145 cells with 1 to 4 mmol/L doses (20). Accordingly, here we also assessed whether IP6-caused induction of p21 and p27 plays any role in its cell death response. As shown in Fig. 3*A*, the percentage of dead cells, assessed by trypan blue staining, increased with IP6 treatment by 2-fold in DU-EV, 2.8-fold in DU-p21, and 2.2-fold in DU-p27 cells,

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but by only 1.1-fold in DU-p21+p27 cells, over their respective controls. These observations, therefore, prompted us to delineate the role of p21 and p27 in IP6-induced apoptosis. We used Annexin V/PI staining to quantify apoptotic cells in DU145 cell variants with or without IP6 treatment. We observed that IP6 increases the apoptotic population by 2-fold ($P < 0.001$) in DU-EV, 2.2-fold ($P < 0.001$) in DU-p21, and 3-fold ($P < 0.001$) in DU-p27 cells; however, the apoptotic effect of IP6 was almost completely lost in DU-p21+p27 cells (Fig. 3*B*). To further verify the relative apoptotic index, we analyzed the levels of cleaved PARP by immunoblot analysis and observed that IP6 increases cleaved PARP levels by 2-fold in DU-EV, DU-p21, and DU-p27 cells but failed to induce any significant PARP cleavage in DU-p21+p27 cells (Fig. 3*C*). Thus, the ablation of either of the CDKIs retains the sensitivity of DU145 cells to IP6-induced apoptosis, however, their simultaneous ablation makes the cells almost completely resistant to apoptosis.

IP6 fails to inhibit DU145 tumor xenograft growth in p21 and p27 simultaneous knockdown condition. All the *in vitro* studies point to the important role of p21 and p27 in inducing IP6-mediated growth inhibition, cell cycle arrest, and apoptosis. To further validate this notion and its significance, we extended our studies to *in vivo* conditions in which DU145 cell variants were inoculated in male athymic nude mice and were fed with IP6 (2%, w/v) in drinking water from day 6 of xenograft implantation, a time at which the tumors were distinctly measurable (3 mm in diameter). Comparing the tumor growth among different DU145 cell variants, DU-p21+p27 cells showed a significantly higher rate of tumor growth as compared with the DU-EV cells, whereas the growth rate of DU-p21 and DU-p27 were slightly higher than that of DU-EV. IP6 treatment slowed down the rate of tumor growth in all the cell variants, except DU-p21+p27 tumors (Fig. 4*A*). After 59 days of IP6 treatment, there was a 44%, 40%, and 46% decrease in tumor volumes in the DU-EV, DU-p21, and DU-p27 groups, respectively; however, no significant effect (only 11% decrease) was observed in the DU-p21+p27 group (Fig. 4*A*). Similarly, IP6 feeding significantly reduced tumor wet weight by 35% in DU-EV (0.6 g/mouse in control versus 0.39 g/mouse in IP6-fed group; $P < 0.05$), 36% in DU-p21 (0.98 g/mouse in control versus 0.62 g/mouse in IP6-fed group; $P < 0.05$), and 50% in DU-p27 (1.09 g/mouse in control versus 0.51 g/mouse in IP6-fed group) tumors (Fig. 4*B*). However, in case of DU-p21+p27, no significant effect of IP6 on tumor weight was observed (1.84 g/mouse in control group versus 1.6 g/mouse in IP6-fed group; Fig. 4*B*). Thus, consistent with the *in vitro* results, IP6 retains its growth arrest efficacy in tumors with knocked down levels of either p21 or p27. However, DU-p21+p27 cells were more tumorigenic than DU-EV cells and did not respond to IP6 treatment, further underscoring the complementary roles of Cip/Kip proteins and their specificity as targets of IP6 efficacy under *in vivo* conditions as well. Western blot analysis of tumor lysates showed that even at the end of the

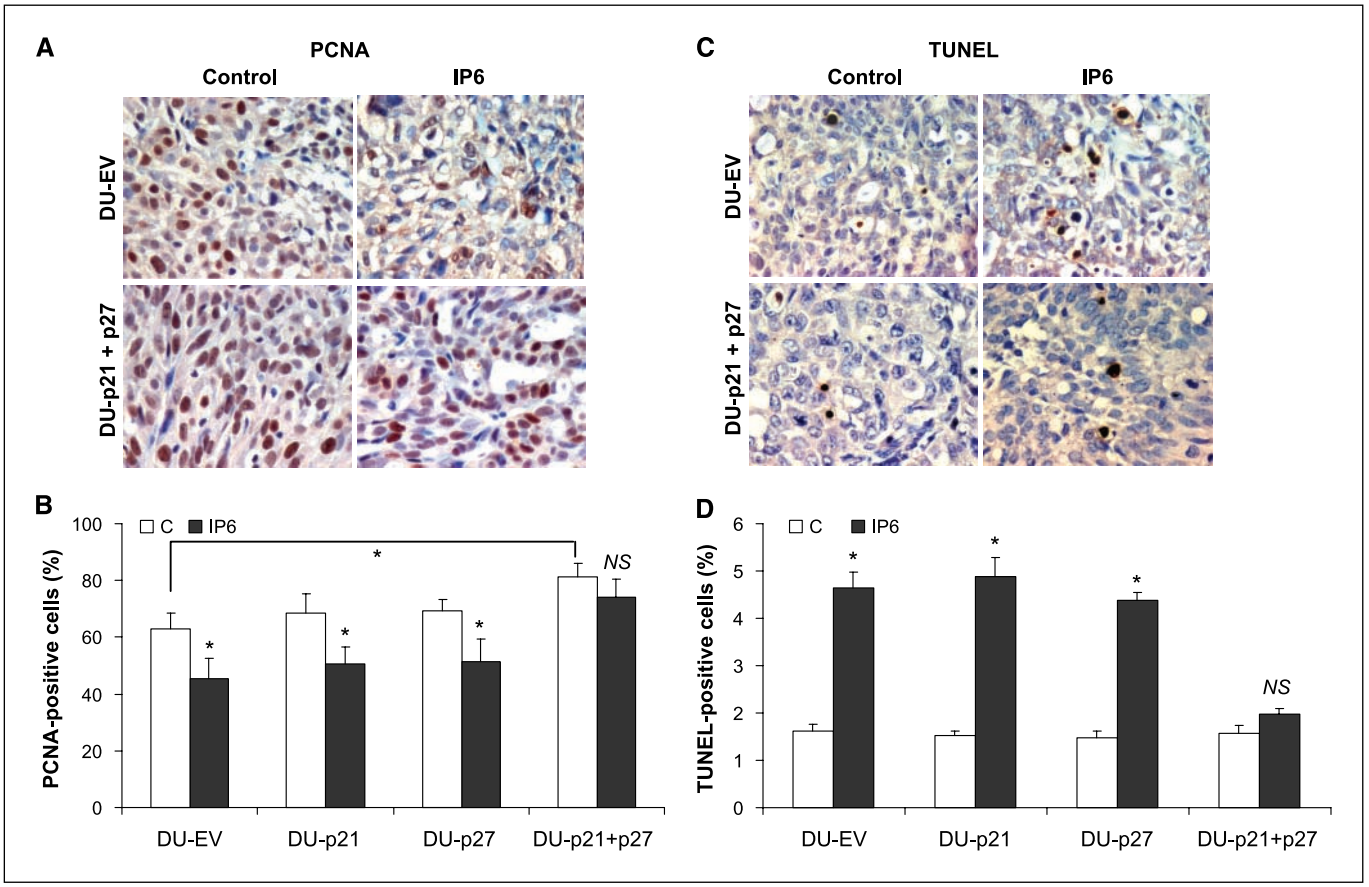

Figure 5. IP6 fails to inhibit cell proliferation and induce apoptosis in DU145 tumors with knocked down levels of p21 and p27. The tumor tissues from the studies detailed in Fig. 4 were analyzed for PCNA and TUNEL staining as detailed in Materials and Methods. Representative images of DU-EV (*A* and *C, top*) and DU-p21+p27 (*A* and *C, bottom*) tumors from control and IP6-fed groups. Immunostaining was quantified for (*B*) PCNA- and (*D*) TUNEL-positive cells as detailed in Materials and Methods. *C,* control; *, $P < 0.001$. *NS,* not statistically significant.

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experiment, the tumors from the DU-p21, DU-p27, and DU-p21+p27 cells had attenuated levels of p21 and/or p27 (Fig. 4*C*), whereas IP6 induced p21 protein expression in DU-EV and DU-p27 tumors, and p27 levels in DU-EV and DU-p21 tumors (Fig. 4*C*). There was no significant difference in body weight, water, and feed consumption between the corresponding control and IP6-fed animals of all the groups indicating the nontoxic property of this phytonutrient (data not shown).

Essential role of p21 and p27 in antiproliferative and proapoptotic effects of IP6 in DU145 tumor xenografts. The *in vivo* antiproliferative and apoptotic effects of IP6 in causing inhibition of PCa xenograft growth and the associated essential role of p21 and/or p27 were next assessed by analyzing the tumor samples for PCNA and TUNEL staining. Inhibition of tumor volume and weight with IP6 administration in the DU-EV group was associated with concomitant reduction in proliferating cells (63% of PCNA-positive cells in control group versus 45% in IP6-fed group; $P < 0.001$; Fig. 5*A* and *B*). Similarly, there was a significant reduction in cell proliferation in the tumor tissues of DU-p21 (from 68% to 50% PCNA-positive cells; $P < 0.001$) and DU-p27 (from 69% to 51% PCNA-positive cells; $P < 0.001$) by IP6 (Fig. 5*B*). However, in case of DU-p21+p27 tumors, the effect was not statistically significant (from 81% to 74% PCNA-positive cells). This differential pattern of inhibition of cell proliferation by IP6 in DU145 cell variant xenografts further corroborates the critical role of Cip/Kip proteins in mediating the antiproliferative efficacy of IP6 under *in vivo* conditions. Quantitative evaluation of apoptosis in tumors showed that IP6 significantly increases the apoptotic population in DU-EV, DU-p21, and DU-p27 tumors by 2.9-fold, 3.2-fold, and 3-fold, respectively (Fig. 5*C* and *D*). However, consistent with our *in vitro* results, IP6 failed to induce any significant apoptosis in DU-p21+27 tumors (1.2-fold induction; Fig. 5*C* and *D*).

Discussion

The novel and central finding in the present study is that p21 and p27, the Cip/Kip family proteins, are indispensable in causing growth arrest and apoptotic death of advanced human PCa DU145 cells by IP6, both *in vitro* and *in vivo*. Furthermore, the simultaneous down-regulation of both p21 and p27 levels results in DU145 cells with a more aggressive phenotype. In this study, we found that the more aggressive DU145 cell variant with knockdown levels of both p21 and p27 was also resistant to IP6-induced cell growth inhibition and apoptosis under both *in vitro* and *in vivo* conditions, thus establishing p21 and p27 as the primary molecular targets of IP6 for its antitumor efficacy against PCa.

Cell cycle progression is governed by the well-orchestrated activation and inactivation of CDKs (29, 30). G_1 to S phase transition plays a crucial role in maintaining the genomic integrity because this phase is critically linked to external stimuli and also commits the cells to DNA replication and subsequent mitosis (31, 32). G_1 checkpoint abrogation is a common phenomenon in carcinogenesis which endows the tumor cells with limitless replicative potential (17, 24). p21 and p27 physically interact with CDK via their amino terminal domain and inhibit CDK kinase activity (29, 30). Thus, activating the G_1 checkpoint by up-regulating the expression of p21 and p27 is a logical approach for controlling cancer cell proliferation. Consistent with this notion, the present study shows that p21 and p27 induction is the central event in IP6-induced G_1 arrest and inhibition of cell proliferation in DU145 PCa cells. More importantly, this study provides the evidence that either p27 or p21 in the absence of p53 and Rb can function in the G_1 to S transition checkpoint and mediate IP6-induced G_1 arrest and cell growth inhibition in DU145 cells (33, 34). Furthermore, in the absence of all four molecules (p21, p27, p53, and Rb) the G_1 checkpoint in response to IP6 is abrogated, resulting in uncontrolled proliferation of PCa cells during IP6 treatment *in vivo* and *in vitro*.

Apoptosis-resistance acquired by cancer cells poses a major barrier to effective treatment (35). Thus, there has been a significant focus on identifying the agents that could induce the apoptotic switch in tumor cells (2, 35). Previously, we have shown that IP6 not only causes G_1 arrest but also induces moderate to high levels of apoptosis in DU145 cells (20). Moreover, it is believed that sustained G_1 arrest eventually drives the cancer cells to apoptosis. In the present study, we investigated the role of p21 and p27 protein levels in mediating the proapoptotic effect of IP6. Although primarily known for their CDK inhibitory activity, there are mixed reports for the role of p21 and p27 in apoptosis induction. For example, adenoviral overexpression of p27 has been shown to induce apoptosis in various epithelial cancer cell lines (36). p27 is also reported to cause tumor necrosis factor receptor 1 accumulation which eventually accounts for its proapoptotic response (37). In case of p21, depending on its subcellular localization and cell type, both proapoptotic and antiapoptotic effects have been reported. For example, nuclear p21 is associated with apoptosis whereas cytosolic p21 is often implicated in resistance to apoptosis (38). From the perspective of chemoprevention studies, the proapoptotic response of various chemopreventive agents has been associated with the concomitant up-regulation of p21 and/or p27 (24, 39). However, most of these studies are correlative and the precise connection between Cip/Kip induction and apoptotic cell death has not often been clearly dissected. In the present study, stable knockdown of Cip/Kip proteins reversed IP6-induced apoptotic cell death, clearly establishing their proapoptotic role, both *in vitro* and *in vivo*. However, the mechanism by which Cip/Kip protein induces apoptosis by IP6 needs further investigation.

An important aspect of this study is that the growth-inhibitory and proapoptotic roles of p21 and p27 observed in cell culture were also retained *in vivo* in xenograft studies. Moreover, in both systems, it was consistently observed that p21 and p27 proteins play compensatory roles because down-regulation of either one of them was not sufficient to reverse the anticancer effects of IP6. Compared with DU-EV tumors, only DU-p21+p27 tumors showed a significantly higher proliferation rate and resistance to apoptosis even with IP6 administration in xenograft studies, thereby emphasizing the critical importance of both these molecules and their relevance to be pursued as prognostic biomarkers of IP6 antitumor efficacy against advanced PCa in which both the tumor suppressor proteins p53 and Rb are nonfunctional (33, 34). Moreover, similar studies are desired in other PCa cell lines differing in tumorigenic grade, as well as in transgenic PCa models, to establish the comprehensive importance of these molecules for antitumor responses of IP6. Nevertheless, the mechanistic aspect and the primary molecular targets identified in this study underscore the critical tumor suppressor functions of p21 and p27 (Cip and Kip family) proteins, and also suggest their potential as biomarkers for tumor progression as well as prognosis (for IP6 efficacy) in clinical cases of PCa (40, 41). Overall, it could be suggested that IP6 is a potential nontoxic, readily available

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chemopreventive dietary agent having potential to control PCa, which also show antitumor response in the presence of either p21 (Cip1) or p27 (Kip1) in PCa cells harboring nonfunctional p53 and Rb; the critical tumor suppressors that are frequently mutated in several types of cancers including PCa. Unlike p53 and Rb, p21 and p27 are not usually mutated in cancer cells (25). This study combined with our previous studies with IP6 in PCa cell culture and animal models, suggest a merit for clinical investigation of IP6 in patients with PCa.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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p21/Cip1 and p27/Kip1 Are Essential Molecular Targets of Inositol Hexaphosphate for Its Antitumor Efficacy against Prostate Cancer

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