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Review

Prostate Cancer and Inositol Hexaphosphate: Efficacy and Mechanisms

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Abstract. *There are now extensive scientific data suggesting the potential role of dietary and non-dietary phytochemicals in the prevention and control of prostate cancer (PCA) growth and progression. PCA is a disease of elderly male populations with a relatively slower rate of growth and progression as compared to most other cancers and, therefore, is a candidate disease for preventive intervention. Overall, PCA growth and progression involve aberrant mitogenic and survival signaling and deregulated cell cycle progression, accompanied by gradual accumulation of genetic and epigenetic changes over a period of years. Several mechanisms, including overexpression of growth, survival and angiogenic factors and their receptors, together with a loss/decrease of tumor suppressor p53, retinoblastoma and cyclin-dependent kinase inhibitor, have been implicated in PCA growth and progression. Therefore, phytochemicals targeting these molecular events could have a promising role in PCA prevention and/or therapy. Inositol hexaphosphate (IP6) is a major constituent of most cereals, legumes, nuts, oil seeds and soybean. Taken orally as an over-the-counter dietary/nutrient supplement, and is recognised as offering several health benefits without any known toxicity. In vitro anticancer efficacy of IP6 has been observed in many human, mouse and rat prostate cancer cells. Completed studies also show that oral feeding of IP6 inhibits human PCA xenograft growth in nude mice without toxicity. In a recently completed pilot study, we observed similar preventive effects of IP6 on prostate tumorigenesis in the TRAMP model. Mechanistic studies indicate that IP6 targets mitogenic and survival signaling, as well as cell cycle progression, in PCA cells. IP6 is also shown to target molecular events associated with angiogenesis. Moreover, IP6 has pleiotropic molecular targets for its overall efficacy against PCA and, therefore, could be a suitable candidate agent for preventive intervention of this malignancy in humans.*

Contents

Abbreviations: IP6, inositol hexaphosphate; PCA, prostate cancer; PIN, prostatic intraepithelial neoplasia; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; Rb, retinoblastoma; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; IGF, insulin-like growth factor; IGFBP-3, IGF-binding protein-3; NF-Î B, nuclear factor kappa B; PARP, poly (ADP-ribose) polymerase; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; TRAMP, transgenic adenocarcinoma of mouse prostate.

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Prostate cancer (PCA) incidence varies widely between countries as well as ethnic populations, due to a combination of various factors, with PCA rates differing up to 90-fold among different populations (1). It has been estimated that dietary factors alone account for up to a 35% difference in cancer rate among different countries (2). Men living in Japan, China, India and other Asian countries are up to ten times less likely to be diagnosed with and die from PCA as compared to those living in the United States (1, 2). Regarding the global incidence of PCA, several epidemiological studies have reported that the incidence of PCA development and the death rate are lower in Asian as compared to Western countries (1-3). These studies have also reported that there is probably a reduced risk of PCA development in Asian countries when compared to Western countries with affluent dietary patterns. The difference in dietary pattern has been identified as one of the major environmental etiologic factors behind such a wide range of variation in PCA incidence and mortality (3-5). The diet in Western countries is based on highly processed foods that are rich in meat, dairy products and refined carbohydrates. Conversely, in Asian countries, the major components of the diet are whole grain cereals, legumes, nuts, vegetables and fruits. The Asian dietary pattern of whole grain and fiber-rich food contains distinctly higher amounts of inositol hexaphosphate (IP6) than that of the Western diet. There is a strong possibility that the lower clinical incidence of PCA and associated mortality in Asian countries, could be, in part, due to these differences in diet. Therefore, the identification of dietary anticancer agents and their inclusion in the regular diet could be helpful in reducing PCA risk and mortality.

Statistics for PCA indicate that it is one of the major epithelial cancers in men and the second most common male malignancy in the United States and European countries (8); it is estimated that 230,110 new cases and 29,900 deaths will occur from PCA in 2004 (8). A study in Detroit, MI, USA, of 600 men, reported that the rate of latent PCA was very high, at 30% for men in their 30s, 50% for men in their 50s and more than 75% for men older than 95 years (9). The disease progression involves autocrine and paracrine growth factor-receptor interactions leading to molecular signaling events, which are the major contributors to the deregulated PCA cell growth and survival (10-12). Deregulated cell proliferation with predominating survival mechanisms are hallmarks for almost every cancer including PCA (13, 14). The hormone-refractory stage of PCA does not respond to androgen-deprivation therapy and also becomes apoptosis-resistant to many cancer chemotherapeutic drugs (15, 16). Therefore, intervention of PCA *via* non-toxic dietary phytochemicals such as IP6, targeting deregulated cell cycle progression and mitogenic and cell-survival signaling, could be a practical and translational (from cell culture and animal studies to humans) approach for the prevention and/or therapy of PCA.

A. IP6: structure and source

IP6 is a six-phosphate derivative of inositol, which has a glucose-like structure. Inositol and its phosphate derivatives are physiologically inter-convertible and are present in the 0.01-1.0 mM range in almost all living cells (7). IP6 is found in abundance in high fiber diets. Most cereals, legumes, nuts, oil seeds and soybean contain 0.5-6.4% (w/w) or even higher levels of IP6 (7). It is also sold as a dietary supplement, either alone or in combination with other antioxidants, and is consumed by many cancer patients for its health benefits. In an animal study, it was reported that physiological levels of IP6 are more dependent on the consumption of an IP6-rich diet than the levels of its lower phytate derivatives (IP5 and IP4) (17). Another study suggested that humans become deficient in IP6 if they consume an IP6-poor diet for as little as two weeks (18).

B. Chemopreventive IP6 efficacy

Consumption of IP6-rich cereals and legumes has been suggested to be associated with reduction in mammary, colon and prostate cancers (19-21). However, the molecular mechanisms of its anticancer effects have not been studied in detail. Various animal studies have revealed that IP6 does not show any noticeable harmful side-effects or toxicity even at higher doses of chronic administration, such as up to 2% w/v or 15 mM in drinking water (19, 20). The presence of IP6 in a variety of foods provides a basis for the scientific evaluation of its anticancer and chemopreventive efficacy.

The beneficial effects of IP6 include prevention against the formation of kidney stones, high cholesterol and heart and liver diseases (22). It also works as an antioxidant by removing free radicals and protects cells from an iron overload by working as an ion chelator (23). Many laboratories have demonstrated the cancer chemopreventive efficacy of IP6 in different animals, as well as in *in vitro* cancer models including prostate, skin, mammary, intestine, colon, lung and liver (21). In the following sections, the anticancer efficacy and associated mechanisms of IP6, with a focus on PCA, are described.

C. *In vitro* anticancer IP6 efficacy

Almost three decades of research on IP6 have revealed its broad-spectrum antineoplastic activities in different cancer models (21). Cell culture studies have indicated that IP6 inhibits the growth of various human cancer cells, including human breast and colon cancer cells (24, 25); suppresses the growth of rhabdomyosarcoma and erythroleukemia cells (26, 27); inhibits the growth and reverses the transformed phenotype of HepG2 liver cancer cells (28); and inhibits cell transformation in mouse epidermal JB6 cells (29). It is

reported that the cancer chemopreventive efficacy of IP6 could be, in part, due to its preventive effect on oxidative DNA damage in cultured cells (30). There are only limited studies showing the anticancer potential of IP6 against different PCA cell lines, and the mechanisms are not completely understood. The details of these studies are described in the following sub-sections.

C1. IP6 efficacy in human PCA cells. The first study showing the *in vitro* anticancer potential of IP6 against PCA was published in 1995 by Shamsuddin and co-workers, who reported that IP6 inhibits growth and induces differentiation of human PCA PC-3 cells (31). In a recent study, IP6 was also reported to inhibit the growth of hormone-refractory human PCA DU145 cells (32). Cell growth inhibition in DU145 cells reached up to 77% after 72 h of 0.25, 0.5, 1, 2 and 4 mM IP6 treatment, in a dose- and time-dependent manner (33). Recently, it has also been shown that IP6 inhibits growth in androgen-dependent human prostate carcinoma LNCaP cells in a dose- and time-dependent manner and that up to 63% growth inhibition occurred after 48 h of 0.5, 1, 2 and 4 mM IP6 treatment (34). IP6 inhibition of EGF and IGF-induced cell growth and proliferation in PC-3 cells has also been observed (unpublished data). In all these PCA cell lines, IP6 also caused significant cell death (32-34). These results clearly demonstrate IP6 efficacy against human PCA cells. More recently, we observed that IP6 did not cause any considerable cell growth inhibition or cell death in non-neoplastic human prostate epithelial PWR-1E cells (unpublished data). Such selective growth inhibitory and cytotoxic effects of IP6 on PCA cells offer great benefits compared to known cancer chemotherapeutic agents for which therapeutic outcomes are compromised by harmful side-effects. The efficacy of IP6 in mouse and rat PCA models is presented below.

C2. IP6 efficacy in mouse PCA cells. PCA growth and progression in TRAMP (transgenic adenocarcinomas of mouse prostate) mice are quite similar to the human form of this disease (35-37). This model is regarded as one of the best available PCA models for chemoprevention or intervention studies (35, 36). Recently, several cell lines, namely TRAMP-C1, TRAMP-C2 and TRAMP-C3, have been established from adenocarcinomas of the prostate developed in TRAMP mice; TRAMP-C1 and TRAMP-C2 are tumorigenic when grafted into syngeneic C57BL/6 hosts (38). By employing TRAMP-C1 cells, the efficacy of IP6 in cell growth inhibition was assessed. In this study, 0.25, 0.5, 1, 2 and 4 mM IP6 resulted in 17-76% cell growth inhibition in a dose- and time-dependent manner, which was also accompanied by an induction of cell death (39).

C3. IP6 efficacy in rat PCA cells. PCA growth and progression in MNU-testosterone-induced rat PCA are quite similar to those in the human form of the disease, specifically in terms of growth dependence on testosterone (40, 41). In addition to the TRAMP model, this model is also regarded as one of the best available models for chemoprevention or intervention studies in PCA (38). Recently, several cell lines, namely H7, I8 and I26, have been established from MNU-testosterone-induced rat PCA, which are tumorigenic when injected sub-cutaneously into syngeneic hosts, and all of them consistently form metastases in the lung and lymph nodes (42). IP6 treatment of both the H7 and I8 cell lines resulted in strong cell growth inhibition in a dose- and time-dependent manner, and was also accompanied by cell death, as seen in other PCA cells (43). Overall, these findings clearly demonstrate the growth inhibitory efficacy of IP6 in human, mouse and rat PCA cells and, therefore, suggest its wider applicability in PCA chemoprevention.

D. *In vivo* anticancer IP6 efficacy

The investigation of the *in vivo* cancer chemopreventive efficacy of IP6 began in the late 1980s. Several studies with animal models have shown that IP6 possesses *in vivo* anti-neoplastic activity against various carcinogen-induced tumors as well as a human cancer cell xenograft in athymic nude mice (21). The first report (1988) indicated that 1% IP6 in drinking water, one week before or two weeks after the administration of azoxymethane, inhibited the development of large intestinal cancer in F344 rats (44). In 1989, it was reported that, in the same animal model, treatment with 2% IP6 in drinking water, even after five months of carcinogen induction, significantly inhibited both the number and size of tumors in the large intestine (19). The same research group also observed that IP6 suppressed large intestinal cancer in another animal model where dimethylhydrazine was used as a carcinogen in CD-1 mice (45). Furthermore, IP6 has been shown to i) inhibit DMBA-induced rat mammary cancer growth (46); ii) regress liver cancer xenotransplant (47); iii) prevent pulmonary adenomas in mice (48); iv) prevent DMBA-induced skin tumorigenesis (49); v) inhibit the growth of rhabdomyosarcoma tumor xenograft growth (26); vi) inhibit growth of mouse fibrosarcoma FSA-1 tumor xenografts in nude mice (50); and vii) inhibit colon carcinogenesis (51, 52). It has also been reported that IP6 treatment reverses colon carcinogen (dimethylhydrazine)-induced suppression of natural killer cell activity, as well as enhancing the baseline natural killer cell cytotoxicity in mice (53).

To the best of our knowledge, there had been no study revealing the *in vivo* efficacy of IP6 against prostate cancer until very recently. For the first time, 1 and 2% (w/v) IP6

feeding in drinking water was observed to inhibit DU145 tumor xenograft growth in athymic nude mice, and this IP6 *in vivo* efficacy was associated with antiproliferative and pro-apoptotic effects in tumor xenografts. Similar effects were observed in a completed pilot study in the TRAMP model. However, there is no study to date showing the efficacy of IP6 in the MNU-testosterone-induced rat prostate carcinogenesis model. A brief description of completed *in vivo* studies in nude mice and the TRAMP model is given below.

D1. IP6 inhibition of human PCA xenograft growth in nude mice. The first *in vivo* efficacy evaluation involved a nude mice xenograft model and showed that IP6 suppresses hormone-refractory human prostate tumor (DU145) growth in athymic nude mice (54). In this study, androgen-independent human prostate carcinoma DU145 cells were injected into nude mice for ectopic tumor xenograft growth, and the mice were assigned to regular drinking water and 1% and 2% IP6 in drinking water (w/v) *ad libitum* for 12 weeks. In this study protocol, IP6 inhibited tumor xenograft growth in a time- and dose-dependent manner, and accounted for 47-66% inhibition in tumor volume/ mouse and 40-66% decrease in tumor weight/ mouse at the end of the study (54). More importantly, IP6 feeding did not show any adverse effect on body-weight gain profiles, or diet and water consumption during the entire study. Immunohistochemical analysis of tumor xenografts showed that tumor growth inhibition was associated with decreased tumor cell proliferation and angiogenesis, and an increased apoptotic cell death in the IP6-treated groups. Together, these findings established the *in vivo* efficacy of IP6 in a pre-clinical prostate cancer model.

D2. IP6 inhibition of PCA growth in the TRAMP model. The TRAMP model is one of the best-characterized transgenic models of prostate carcinogenesis (36, 37). TRAMP mice develop spontaneous autochthonous invasive carcinomas of the dorsolateral and ventral prostate at an essentially 100% rate within 18-24 weeks (36). The TRAMP model mimics several characteristics of human prostate cancer in that the mice develop early high-grade prostatic intraepithelial neoplasia that usually develops around 12 weeks, which then progresses into tumor formation and eventually metastasizes to lymph nodes and bones. Several recent investigations have employed the TRAMP model for prostate cancer chemoprevention efficacy studies. Tests on this model have revealed that: R-flurbiprofen, a non-steroidal anti-inflammatory drug, inhibits progression of PCA; flutamide prevents primary PCA; DFMO prevents prostate carcinogenesis; dietary genistein reduces the incidence of poorly-differentiated prostatic adenocarcinoma; toremifene, an anti-estrogen, prevents PCA; and that oral infusion of green tea polyphenols inhibits prostate carcinogenesis (55-60).

The efficacy of IP6 in the TRAMP model is the basis of our on-going study (unpublished data), in which oral feeding of IP6 was found to inhibit PCA growth without any apparent sign of toxicity. Four-week-old TRAMP male mice were fed with 2% (w/v) IP6 in drinking water for 20 weeks, and 34% ($p<0.05$) inhibition in PCA growth was observed by measuring the weight of the genitourinary (GU) system (prostate + prostate tumor + seminal vesicle + bladder) as compared to those TRAMP mice who were kept on regular drinking water, without any apparent sign of toxicity as monitored by body weight gain, diet and water consumption. The possible adverse effects of IP6 feeding on normal prostate in the parental C57BL/6 strain of TRAMP model were also examined. No significant difference in GU weight was observed in the mice kept on either regular drinking water (control) or 2% IP6 in drinking water for 20 weeks, and no considerable changes in body weight gain, diet and water consumption were recorded. Data analysis revealed that IP6 inhibited the abnormal increase in GU weight by 48%. These findings are the primary indications that oral IP6 inhibits PCA growth in the TRAMP model without any apparent toxicity, as observed in the human PCA xenograft model mentioned above.

Overall, these studies suggest that IP6 has chemo-preventive efficacy in several different animal models of carcinogenesis, and that it inhibits human PCA DU145 xenograft growth in nude mice and prevents prostate tumorigenesis in the TRAMP model. Some studies have also evaluated the PCA chemopreventive efficacy of test agents in the MNU-testosterone-induced Wistar rat PCA model, characterized by androgen-promotion, a reasonable latency period and the tendency to develop prostatic intraepithelial neoplasia (PIN) in addition to invasive carcinomas of the prostate-seminal vesicle complex (40, 41). In order to get a comprehensive overview of IP6 efficacy in PCA prevention and to form a basis for clinical trials in human PCA patients, future studies are needed to evaluate IP6 efficacy in the MNU-testosterone-induced rat prostate tumorigenesis model.

E. Inhibitory effect of IP6 on PCA cell cycle progression

Inhibition of deregulated cell cycle progression by many anticancer agents is one of the primary mechanisms to inhibit the growth of cancer cells. There are many checkpoints in the mammalian cell cycle in which G1-S and G2-M play a crucial role in the regulation of cell cycle progression (61, 62). G1 arrest can prevent the replication of damaged DNA or allow enough time for DNA repair, and G2 arrest is known for stopping cells with unrepaired DNA damage from entering into or cycling through the mitotic phase (62). These checkpoints are helpful in checking the uncontrolled proliferation of cancer cells by making them cytostatic, terminally-differentiated or by inducing apoptotic death.

Completed studies have shown that IP6 invariably causes G1 arrest in PCA cells (33, 34, 39, 43). Consistent with cell growth inhibition, 0.25, 0.5, 1, 2 and 4 mM IP6 for 24 h resulted in 51-60% cells in G1-phase as compared to the control with only 38% cells in G1, at the expense of both S-phase as well as G2-M-phase cell populations in DU145 cells (33). A similar dose-dependent effect on G1 arrest by IP6 was observed in human PCA LNCaP and mouse PCA TRAMP-C1 cells (34, 39). In the case of rat PCA H7 cells, IP6-induced G1 arrest was at the expense of both S- and G2-M-phase cell populations, while in H8 cells it was only at the expense of the S-phase cell population (43). IP6 treatment of non-neoplastic prostate epithelial PWR-1E cells did not show any considerable effect on cell cycle progression (unpublished data). This selective effect on G1 arrest constitutes an important mechanism of the growth inhibitory effect of IP6 in PCA cells.

E1. Effect of IP6 on cyclin-dependent kinase inhibitors. Several studies have revealed the induction of G1 arrest and cell growth inhibition, together with an up-regulation of the cyclin-dependent kinase inhibitors (CDKIs) Kip1/p27 and/or Cip1/p21, in many types of cancer cells including PCA (63-73). These reports highlight the importance of these CDKIs as favorable drug targets which mediate the antineoplastic effects of many potential agents. The Kip and Cip family of proteins share sequence homology and can bind to the same types of cyclin/CDK, but with different affinities (14). *Kip1/p27* and *Cip1/p21* genes are located on different chromosomes in human and mouse (74-76), however they share sequence homology (74-76). The screening of human tumors, including prostate tumors, has shown frequent mutations in *Kip1/p27*, including the *p53* and retinoblastoma gene; however, such phenomena are less common in *Cip1/p21* (77-79). The promoter of the *Cip1/p21* gene contains a p53-binding site and therefore mediates the growth inhibitory, cell cycle or apoptotic effects of p53 (80, 81). However, *Cip1/p21* expression is also regulated by a p53-independent mechanism (82, 83). Cip1/p21 is shown to bind with PCNA and to interfere with other factors in replication complex assembly, which subsequently controls DNA replication (84, 85).

The major function of Kip1/p27 and Cip1/p21 is to bind and inhibit the kinase activity of cyclin-CDK complexes, resulting in the alteration of phosphorylation events that subsequently control the checkpoints during cell cycle progression (61, 62). Some recent studies, however, have also suggested CDK-independent functions of these CDKIs, such as potential assembly factors, apoptosis regulators, transcriptional cofactors, and in cell migration (86). Mostly, in the advanced stage of PCA, both *p53* and *Rb* genes are mutated, but *Kip1/p27* and *Cip1/p21* are functional though their expressions are suppressed (77-79). It has been observed that decreased Kip1/p27 expression is associated with aggressive phenotype of prostatic carcinomas, and the loss of Cip1/p21 function results in the failure of radiation response in PCA patients (87). CDKIs have also been shown to control the cell cycle in response to various stimuli, including growth inhibitory signals (88). Therefore, Kip1/p27 and Cip1/p21 present logical targets in tumor cells with *p53*, *Rb*, and/or other tumor suppressor gene mutations.

Studies on human PCA cells have indicated that IP6 strongly increases Kip1/p27 (up to 9-fold) and Cip1/p21 (up to 3-fold) protein levels in a dose-dependent manner (33, 34). DU145 cells do not have functional p53, therefore, IP6-caused induction of Cip1/p21 in DU145 cells involves p53-independent mechanisms which are yet to be defined (33). An IP6-induced increase in Kip1/p27 and Cip1/p21 in human PCA cells has been reported to be accompanied by their increased physical interaction with CDKs and cyclins, thereby causing a decrease in the kinase activity associated with CDKs and cyclins (33, 34). However, more studies are needed to further define the role of IP6-induced CDKIs in G1 arrest in PCA cells. In this direction, our ongoing study with small interfering RNA for Kip1/p21 and Cip1/p21 revealed that either of the *Kip1/p21* and *Cip1/p21* functional genes is sufficient for mediating IP6-induced G1 arrest in DU145 cells (unpublished data).

E2. Effect of IP6 on cyclin-dependent kinases and cyclins. CDKs are catalytic subunits and require regulatory subunit cyclins for their kinase activity, which is critical for cell cycle progression through checkpoints (61, 62, 89). They are generally regulated by growth responsive mitogenic stimuli; however, in cancer cells, due to constitutive mitogenic signaling, they are overexpressed and remain activated, causing deregulated cell cycle progression and uncontrolled cell proliferation (90, 91). In addition, modulation of cell cycle regulators, leading to growth induction/inhibition, has also been linked to alterations in mitogenic signaling (63, 73, 90). CDK2, CDK4 and CDK6 are important G1 CDKs; CDK4 and CDK6 are associated with cyclin D and regulate early G1, while CDK2 associates with cyclin E for late G1, and with cyclin A for late G1- to S-phase transition (61, 62, 89). The increased expression of G1 cyclins in cancer cells is known to provide them with an uncontrolled growth advantage, as most cancer cells either possess non-functional CDKI or have low CDKI expression (62). CDKs and cyclins have been suggested as critical targets in controlling cell cycle progression in cancer cells by anticancer agents. Many chemopreventive agents have been shown to decrease CDK2, CDK4, CDK6, cyclin D1 and cyclin E expressions as well as inhibiting their kinase activities, thereby arresting cancer cells in G1-phase (63, 73).

Investigations on DU145 cells have indicated that IP6 does not have any considerable effect on the protein levels

of G1 CDKs and associated cyclins; however, it does inhibit kinase activity associated with these CDKs and cyclins (33). It has been observed that IP6 increases the binding of Kip1/p27 and Cip1/p21 with cyclin D1 and cyclin E, causing a concomitant inhibition in kinase activities associated with CDK2, CDK4, CDK6, cyclin D1 and cyclin E (33). Similarly, in LNCaP cells, an IP6-caused increase in Cip1/p21 and Kip1/p27 was associated with their increased binding to CDK2 and CDK4, as well as with a strong decrease in their kinase activity (34). However, IP6 also decreased CDK4 and cyclin D1 levels in LNCaP cells.

E3. Effect of IP6 on the Rb-E2F family of proteins. In addition to p53, Rb and Rb-related proteins are important players in cell cycle regulation regarding control of the G0-G1 and G1-S check-point transition (13, 14). The Rb family members, including pRb (p110) and the pRb-related proteins pRb/p107 and pRb2/p130, are nuclear phosphoproteins, and are regulated in a cell cycle-dependent manner by phosphorylation (91, 92). These are critical targets for inactivation by transforming oncoproteins (92, 93). The hypophosphorylated forms of these proteins bind to and modulate the activity of the E2F family of transcription factors, known to induce the transcription of genes needed for cell cycle progression (93, 94). The pRb2/p130-E2F4 complex is the most abundant E2F complex, which maintains a state of transcriptional silence during G0-phase (95), suggesting that the agents which induce cell cycle arrest by overcoming the low expression and/or loss of function of tumor suppressor genes could be useful for both human PCA prevention and therapy (73).

IP6 has been reported to increase hypophosphorylated levels of pRb in LNCaP cells and pRb-related proteins in DU145 cells. IP6 also increased binding of these proteins with E2Fs, thereby decreasing the transcriptionally active free E2Fs level. This effect of IP6 could be attributed to a decreased expression of growth responsive genes and subsequent growth inhibition of PCA cells *via* G1 arrest in cell cycle progression (Figure 1). The decreased phosphorylation of the Rb family proteins is a consequence of the inhibitory effect of IP6 on CDK-cyclin kinase activity (33, 34). E2Fs are down-stream targets for the action of pRb family proteins (94). In DU145 cells, due to loss of pRb and p53, the transcriptional activity of E2Fs is very high, playing a critical role in deregulated cell cycle progression, a common phenomenon in tumor growth and progression (92, 94). In this regard, IP6 increased hypophosphorylated levels of pRb/p107 and pRb2/p130 in DU145 cells, and caused a moderate decrease in E2F4 level (33). IP6 also increased the binding of E2F4 with pRb/p107 and pRb2/p130, thereby decreasing the free E2F4 level for transcriptional activity, which could be linked to the growth inhibitory response of IP6 through cell cycle arrest (33). Consistent with a decrease in CDK-cyclin kinase activity in LNCaP cells, IP6 increased hypophosphorylated levels of Rb with a decrease in Rb phosphorylation at serine 780, 807/811 sites, and also caused a moderate to strong decrease in E2F1, E2F4 and E2F5 levels (34). Overall, these effects of IP6 suppress the E2F transcriptional activity and lead to a G1 arrest in PCA cell cycle progression, thereby establishing the modulatory effect of IP6 on CDKI-CDK-cyclin and Rb-E2F complexes for G1 arrest in PCA cells (Figure 1).

F. IP6 inhibits mitogenic signaling in PCA

PCA growth and progression are accompanied by enhanced autocrine expression of growth factors and their receptors, and these growth factors have been shown to greatly enhance mitogenic responses in PCA cells (10-12). Growth responses, including survival, are generally mediated *via* activation of EGF and IGF family receptors, leading to subsequent activation of MAPK and PI3K/Akt which are constitutively active in PCA (10-12, 96-98). Advanced human PCA expresses high levels of EGFR and growth factors (*e.g.* transforming growth factor α, TGFα), which leads to an autocrine loop for autonomous PCA cell growth, proliferation and metastasis (90). EGFR activation causes its dimerization, which recruits the membrane-bound clathrin-associated protein complex-2 (AP-2) to further transmit the signal through the Shc-Grb2-Ras-Raf-MAPK pathway (10-12, 99). It has been reported that PCA progression from androgen-dependent to advanced hormone-refractory stage is associated with an increased activation of ERK1/2 (100). ERK1/2 is a converging point for mitogenic signals coming from the membrane receptor, as well as from non-receptor-mediated mitogenic signaling (100). Epidemiological studies have also suggested that a higher ratio of circulating IGF-1 to IGFBP-3 levels increases the risk of PCA (101-103). IGFs and IGFBPs are important regulators of IGF-receptor activation (104). Increased plasma levels of IGFBP-3 sequester IGFs and inhibit IGF receptor signaling, which could reduce PCA risk and progression (104, 105). Therefore, inhibition of these signaling events could form an important strategy in the intervention of human PCA growth and progression (Figure 2).

The results of our completed study indicate that IP6 inhibits TGFα-induced EGFR signaling in DU145 cells (32). In this study, IP6 impaired the endocytosis of activated EGFR by inhibiting its binding to AP-2. Further downstream, IP6 inhibited the phosphorylation of Shc, as well as its association with EGFR. Subsequently, strong inhibition of TGFα-induced ERK1/2 and PI3K-Akt activation was also observed. Inhibition of fluid phase endocytosis of EGFR was associated with inhibition of the PI3K-Akt pathway by IP6. These findings also suggested that AP2 itself might act as a

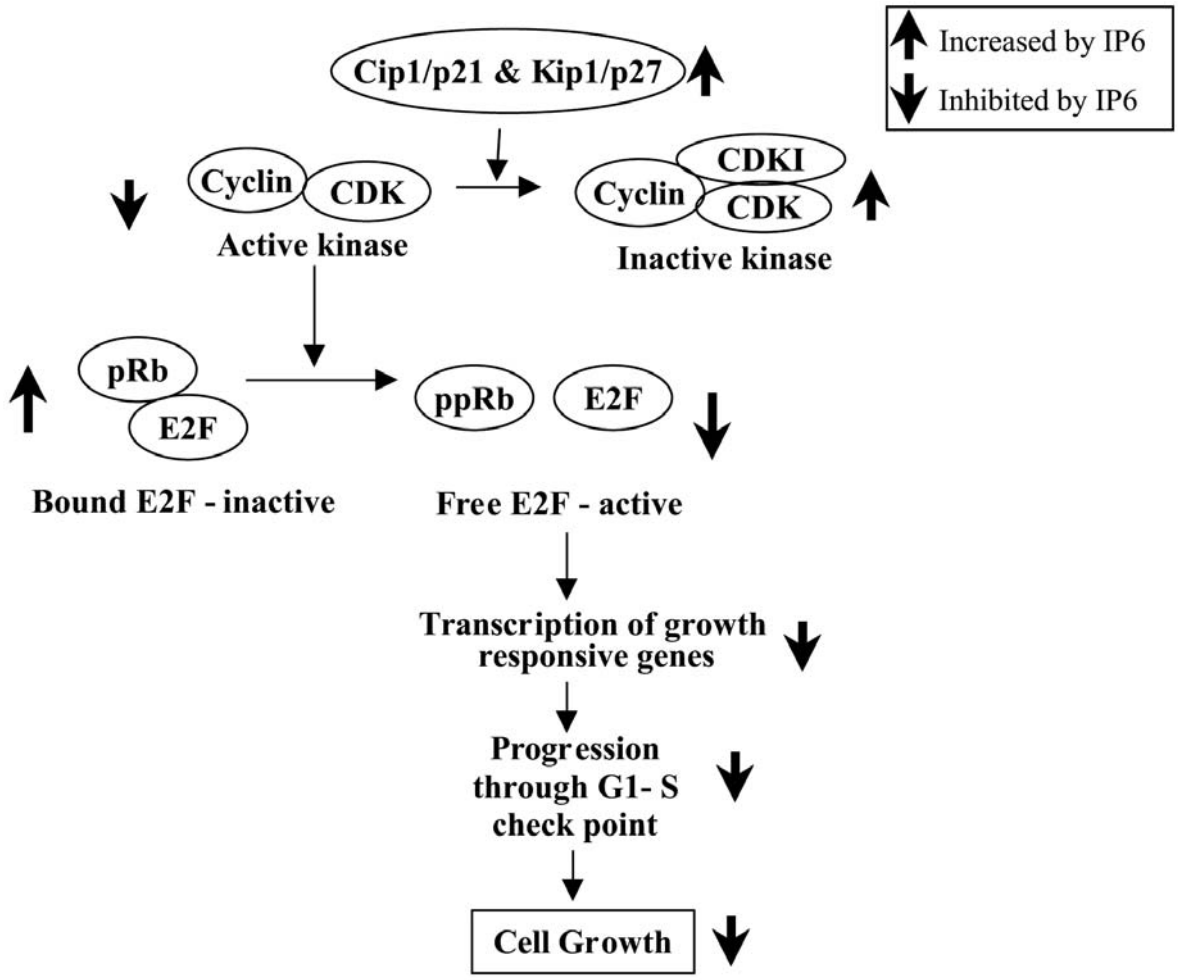

Figure 1. *Effect of IP6 on G1 cell cycle regulators leading to G1 arrest and cell growth inhibition in PCA cells. CDK in association with cyclin forms an active kinase complex, which phosphorylates pRb to release E2F (free), needed for the transcriptional activation of growth responsive genes and subsequent progression of cells through the G1-S check-point. CDKI (e.g. Cip1/p21 or Kip1/p27) is known to physically interact with CDK-cyclin complex to block its kinase activity. Bold upward and downward arrows indicate increasing/enhancing and decreasing/inhibitory effects of IP6, respectively.*

receptor-binding site for IP6 in DU145 cells. We have also observed that IP6 inhibits EGF and IGF-1-induced activation of AP-1 transcription factor in human PCA cells (unpublished data). In a DU145 tumor xenograft study, 1-2% IP6 feeding resulted in a 27-53% decrease in proliferation index, as examined by PCNA immunostaining (54). In this study, IP6 was also found to cause an increase in IGFBP-3 secretion from tumors, perhaps by inhibiting mitogenic IGF receptor signaling by sequestering IGFs in circulation. Since PCNA is one of the molecular targets of mitogenic signaling, an *in vivo* inhibitory effect of IP6 on these signaling events might be, in part, responsible for the overall growth inhibitory efficacy of IP6 in PCA. Further studies are needed to define the involvement of these pathways and molecular targets in the antiproliferative efficacy of IP6 in PCA.

G. IP6 inhibits cell survival signaling in PCA

Deregulated growth of malignant cells is further combined with the loss of apoptotic responses, as most cell survival and anti-apoptotic events are constitutively active in PCA cells (10-14, 106). Accordingly, in addition to inhibition of uncontrolled cell growth, an induction of apoptotic death of PCA cells is an important aspect in controlling this malignancy. Therefore, agents having both growth inhibitory as well as apoptotic efficacy could be especially effective in PCA prevention and therapy. Programmed cell death can be induced by many factors leading to activation of the caspase pathway and execution of apoptosis (107-110). Caspase (a family of cysteine proteases) activation and poly (ADP-ribose) polymerase (PARP) cleavage are regarded as

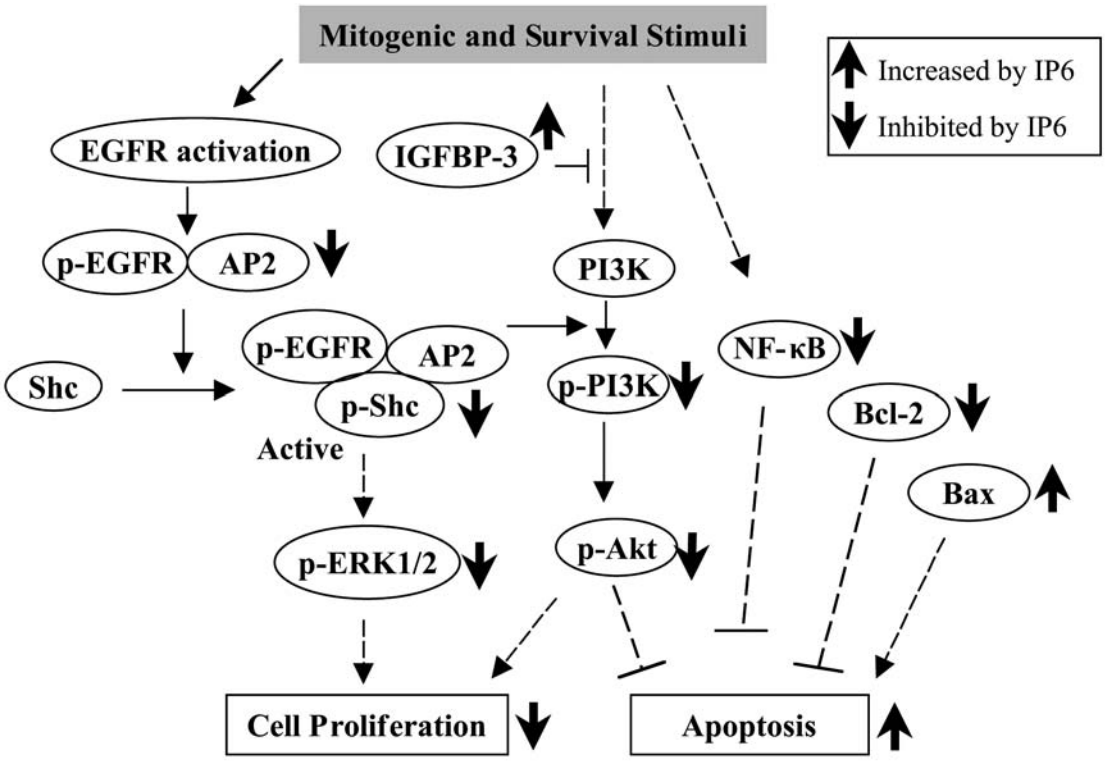

Figure 2. *Proposed mechanisms of IP6 action on mitogenic and cell survival signaling in PCA. Mitogenic and survival stimuli activate EGF and IGF receptor signaling for cell proliferation and survival. Activated EGFR recruits AP2 and then Shc to form an active complex and transmits mitogenic signals downstream through mitogen-activated protein kinases (e.g. ERK1/2) or through the PI3K-Akt pathway. Survival stimuli activate PI3K-Akt and NF-Î B pathways, and increase anti-apoptotic proteins (e.g. Bcl-2) and decrease pro-apoptotic proteins (e.g. Bax) to inhibit the apoptotic death of cancer cells. IGFBP-3 is an antagonist for IGF receptor signaling and is known to inhibit cell proliferation and cause apoptosis in PCA cells. Bold upward and downward arrows indicate increasing/enhancing and decreasing/inhibitory effects of IP6, respectively.*

relevant biomarkers in apoptosis induction (110, 111). Loss of mitochondrial integrity and subsequent release of cytochrome c has been linked to the initiation and activation of some apoptotic cascades (112, 113). The released cytochrome *c* is complexed with Apaf-1 and pro-caspase 9 (dATP-dependent) to form the 'apoptosome', which releases activated caspase 9 to further initiate the activation of the caspase cascade, leading to apoptotic biochemical and morphological changes (114-116).

Consistent with these reports, we have observed that IP6 inhibits growth (discussed earlier) as well as inducing apoptotic death in LNCaP and DU145, as well as in TRAMP-C1 cells, in a dose- and time-dependent manner (33, 34, 39). We have observed similar apoptotic effects of IP6 in PC-3 cells (unpublished data). An *in vivo* apoptotic effect of IP6 feeding has also been observed in a DU145 tumor xenograft study (54). TUNEL analysis of the tumor xenograft showed a dose-dependent increase (2-4 fold) in apoptotic index by 1-2% IP6-feeding (54). Together, these results convincingly suggest both the *in vitro* as well as *in vivo* pro-apoptotic efficacy of IP6, and thus the potential contribution of IP6 to the inhibition of PCA cell growth in culture and prostate tumor growth *in vivo*. Mechanistic studies have shown the activation of caspases and PARP cleavage by IP6 in apoptosis induction in PCA cells (33, 39). However, in TRAMP-C1 cells, IP6-induced apoptotic cell death involved both caspase-dependent and caspase-independent mechanisms (39). In this study, 2 mM IP6 induced up to a 14-fold increase in apoptotic cell death and up to a 6-fold increase in caspase 3 activity, while Pan caspase inhibitor could only reverse about 50% of IP6-induced apoptosis with complete inhibition of caspase 3 activity, thereby providing convincing evidence of both caspase-dependent and -independent mechanisms in IP6-induced apoptosis of TRAMP-C1 cells.

The balance between proliferation, growth arrest and apoptosis in tumors has been associated with patient survival and clinical outcome. The results of several studies suggest that apoptosis frequently occurs in cells at G1-phase of the cell cycle and arrest in late G1- or S-phase can accelerate apoptosis (117-120). Based on these reports, it could be suggested that IP6-induced G1 arrest in PCA cells

might be, in part, responsible for their apoptotic death. In other apoptotic mechanisms, Bcl-2 family members consisting of both pro-apoptotic (such as Bcl-2) and anti-apoptotic (such as Bax) proteins, are considered major players, with the balance and interaction between them determining the fate of the cell (106, 121, 122). In cancer cells, the equilibrium is shifted towards the anti-apoptotic end, making these cells resistant to apoptotic death. It has been observed that IP6 increases the ratio of Bax to Bcl-2 in LNCaP cells, suggesting their possible role in mitochondrial apoptosis (34). Further, it has also been observed that IP6-induced apoptosis was associated with a moderate inhibition of the constitutively active NF-Î B pathway in DU145 cells (123); it was, however, up-regulated in serum-starved PC-3 cells (unpublished data). In another study, IP6 inhibited ligand-induced activation of PI3K-Akt signaling, also constitutively active in many PCA cells (32). These two pathways are major cell survival mechanisms in cancer cells and are also known to contribute to inducible drug resistance in many cancer cells. In the previously mentioned tumor xenograft study, IP6 increased levels of IGFBP-3, which is known to sequester IGFs and inhibit IGF receptor-mediated survival signaling (104, 105). However, IGFBP-3 is also known to induce apoptosis *via* an IGF receptor-independent pathway (124). Overall, these studies suggest that IP6 has multiple molecular targets in PCA for inhibiting their survival *via* apoptosis induction (Figure 2).

H. IP6 inhibits tumor angiogenesis in PCA

Angiogenesis is the formation of new blood capillaries from the pre-existing vasculature, and involves endothelial cell growth, proliferation, invasion and migration (125, 126). It is regulated by an equilibrium between pro- and anti-angiogenic factors, which is shifted towards the pro-angiogenic environment during tumor angiogenesis (125). Vascular endothelial growth factor (VEGF) is a critical angiogenic factor, which is known to accelerate the majority of the biochemical events needed for neo-angiogenesis (127). A proper blood supply is essential for the growth of tumors beyond a critical avascular size limit, as well as for metastasis (125-127). Tumors are known to remain quiescent and localized for years, suggesting the possible role of nutritional factors in keeping avascular lesions dormant over time (125, 126, 128, 129). This argument could be supported by the fact that dietary factors have an important regulatory impact on our cellular physiology and homeostasis and could also influence the balance between pro- and anti-angiogenic factors. Therefore, identification of dietary factors with direct or indirect anti-angiogenic effects could form a logical strategy for the prevention of cancer growth and metastasis (130, 131). In this regard, IP6 has shown considerable angiopreventive efficacy, as described below.

In the DU145 tumor xenograft study described earlier, it was observed that IP6-feeding inhibited microvessel density in tumors (54). Immunostaining of tumor sections for CD31 revealed that the inhibition in microvessel density was 24% and 53% by 1% and 2% (w/v) IP6-feeding in drinking water, respectively (54). In this study, IP6 was also found to cause a 53-65% decrease in the tumor-secreted level of VEGF in mouse plasma. These findings suggested that a decrease in VEGF secretion from tumors could be one of the possible anti-angiogenic mechanisms of IP6-caused decrease in prostate tumor xenograft vasculature. In a recently published study, it was reported that IP6 inhibits the growth of bovine aortic endothelial cells, suppresses capillary tube formation and disrupts capillary tubes pre-formed by human umbilical vein endothelial cells on matrigel (132). IP6 has also been shown to inhibit basic fibroblast growth factor-induced vessel formation in a matrigel plug assay (132). In this study, the authors also observed an inhibitory effect of IP6 on VEGF expression and secretion in liver cancer cells. Overall, these reports suggest the involvement of both direct and indirect anti-angiogenic effects of IP6, which could potentially inhibit tumor angiogenesis and prevent tumor growth and metastasis.

Conclusion

In summary, the naturally occurring carbohydrate IP6 possesses mechanism-based *in vitro* as well as *in vivo* anticancer efficacy against PCA. More importantly, oral IP6 is found to be non-toxic in animal studies as well as to non-neoplastic human prostate epithelial cells in culture. Additionally, the vast experimental evidence for the antineoplastic efficacy of IP6 in various other cancers supports its merit for clinical development as a cancer chemopreventive agent. It could be suggested that dietary intervention of PCA by IP6 might be useful in slowing down the growth and progression of the disease in aging males, which would reduce the associated morbidity and mortality, as well as the burden of PCA management on health care systems. IP6 could also be clinically relevant for the human population at high risk of developing PCA as well as those with different stages of this malignancy. Overall, based on its properties, including non-toxicity, high efficacy, low cost and human acceptability, we suggest the promise and potential of IP6 as an ideal preventive and/or therapeutic agent against prostate cancer.

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