Mechanisms of action of novel agents for prostate cancer chemoprevention

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Abstract

Despite advances in the understanding of prostate cancer (PCa) growth and development, it is still the leading incidence of cases and the second leading cause of mortality due to cancer in men. The problem of early diagnosis compounded with the emergence of androgen independence during commonly used anti-androgen therapy of PCa, have been discouraging for optimal therapeutic response. Recently, many chemopreventive agents, including silibinin, inositol hexaphosphate, decursin, apigenin, acacetin, grape seed extract, curcumin, and epigallocatechin-3 gallate have been identified in laboratory studies, which could be useful in the management of PCa. In vivo pre-clinical studies have indicated chemopreventive effect of many such agents in PCa xenograft and transgenic mouse models. The molecular targets of these agents include cell signaling, cell-cycle regulators, and survival/apoptotic molecules, which are implicated in uncontrolled PCa growth and progression. Furthermore, angiogenic and metastatic targets, including vascular endothelial growth factor, hypoxia-inducing factor-1α, matrix metalloproteinase, and urokinase-type plasminogen activator are also modulated by many chemopreventive agents to suppress the growth and invasive potential of PCa. This review focuses on novel PCa chemopreventive observations in laboratory studies, which could provide the rationale for the prospective use of chemopreventive agents in translational studies.

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Introduction

Prostate cancer (PCa) is a chronic disease, and therefore, its chemoprevention is emerging as an attractive additional strategy for disease control. In recent years, considerable progress has been made in this direction, which has led to the identification of novel cancer chemopreventive agents and their mode of action. Since the existing therapies for PCa have done little in the improvement of morbidity and mortality, the clinical application of chemoprevention should be explored enthusiastically. Several molecular pathways and various stages of cancer could be the targets for chemoprevention. However, according to most researchers, cancer chemoprevention includes prevention, suppression and/or reversal of early and/or late stages of cancer growth, and development by ideally non-toxic natural or synthetic agents.

PCa behavior is mostly unpredictable; however, it usually takes longer time for progression to malignancy and metastasis and, therefore, provides a broader window for its management, including the suitability for chemopreventive intervention. Whereas, it is likely that chemoprevention modality of cancer management would not necessarily eliminate the lesions, it is expected to halt neoplastic progression of pre-neoplastic lesions that certainly would improve morbidity and survival time in PCa patients. As described in detail later in the clinical studies section, PCa certainly provides this opportunity where several chemoprevention trials in patients start at pre-neoplastic condition known as high-grade prostatic intraepithelial neoplasia (PIN) and the positive outcomes are measured as reduction in clinical PCa.

Life style and dietary habits have been identified as major risk factors in PCa growth and progression (Whittemore et al. 1995, Potter & Steinmetz 1996, Clinton & Giovannucci 1998, Abdulla & Gruber 2000, Agarwal 2000). Epidemiological data indicate that vegetables and fruits containing chemopreventive agents could have protective effect against cancer...
Potential PCa chemopreventive agents

Usually, PCa patients live with the disease for many years until it becomes metastatic and, therefore, present an opportunity for chemopreventive intervention. There is also a wide variation in PCa incidence among different ethnic populations, wherein dietary habit has been identified as one of the major etiologic factors (Clinton & Giovannucci 1998, Abdulla & Gruber 2000). Epidemiological studies suggest that about two-thirds of cancer can be prevented by incorporating healthy agents in, and reducing those, which increase cancer risk from the diet (Clinton & Giovannucci 1998, Abdulla & Gruber 2000). However, due to lack of sufficient pre-clinical and clinical studies, it has been difficult to generalize such statement for the human population. Nonetheless, the major advantage of chemoprevention as compared with other forms of treatment is the lack of systemic toxicity. Another advantage of chemoprevention is recent laboratory findings showing that when used in combination, chemopreventive agents improve the therapeutic response of other PCa treatment modalities, such as anti-androgen, chemotherapeutics, or radiotherapy in rodent models or cell culture studies (Tyagi et al. 2002, Deeb et al. 2003, Chengil et al. 2004, Venkateswaran et al. 2004, Narayanan et al. 2005). Therefore, in the present circumstances, chemopreventive agents could be used as an alternative, as well as a combination treatment option in the management of PCa. In the following sections, we have described the emerging PCa chemopreventive agents with their potential molecular targets and clinical implications where applicable.
cell-culture studies. An account of such chemopreventive targets in PCa has been described below.

**Targets for PCa chemoprevention**

The mechanism-based efficacy without any considerable side effect is an essential requirement for the clinical development of a cancer chemopreventive agent. In PCa, often, ligand-induced signaling drives cell proliferation and survival, which are accompanied by deregulated cell-cycle progression (Jones et al. 2001, Fernandez et al. 2002). Many signaling pathways are constitutively active that lead to persistent growth and progression in PCa (Gioeli et al. 1999). In this fashion, PCa cells keep accumulating neoplastic genetic and epigenetic changes leading to metastatic disease. Furthermore, neo-angiogenesis has been observed as an obligatory requirement for the solid tumor growth, including prostate tumors (Lara et al. 2004, Cox et al. 2005). A summary of these PCa chemopreventive targets is included in Fig. 1. These neoplastic biological events are regulated by sequential activation and deactivation or expression and suppression of many cellular molecules, which could be targeted by chemopreventive agents to intervene with the tumor growth and progression. It is important to briefly mention here that apart from these mechanisms, natural chemopreventive agents also possess anti-oxidant activity and can inhibit inflammation and stimulate phase II detoxification enzyme activity. Further, there are other molecular targets, such as fatty acid synthase, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), as well as stromal and epithelial cell interaction and associated paracrine regulatory mechanisms, which could be modulated by many chemopreventive agents in their overall cancer preventive efficacy. However, in the following sections, we have focused on those chemopreventive targets, which are summarized in Fig. 1.

**Cell signaling targets**

**Androgen receptor (AR) signaling**

The steroid enzyme 5α-reductase converts testosterone into dihydrotestosterone (DHT), an active androgen, which binds to AR leading to its nuclear translocation for the transcriptional activation of androgen-responsive genes (Sommer & Haendler 2003; Fig. 2). In the beginning, PCa growth is primarily regulated by androgens, and therefore, androgen ablation therapy is carried out as the first line of treatment; however, PCa relapses within a few years and at this stage, the...
malignancy is androgen independent (Feldman & Feldman 2001, Sommer & Haendler 2003). Recent studies have suggested that various approaches employed in androgen ablation therapy do not cause a complete depletion of androgens (Scher & Sawyers 2005). In such conditions, even nanomolar concentrations of testosterone and DHT, most likely produced by adrenal glands or tumor itself, are sufficient for the transactivation of AR to support PCa cell proliferation (Mohler et al. 2004, Titus et al. 2005). AR gene amplification accompanied by an increased AR mRNA and protein production had been frequently observed during the relapse of the disease after hormone ablation therapy (Feldman & Feldman 2001, Edwards et al. 2003, Ford et al. 2003, Sommer & Haendler 2003). This could be an adaptive response of the tumor cell to become sensitive to low concentrations of the androgens. Furthermore, AR mutations have been observed in up to 50% of the PCa tissue samples, which are mostly in the ligand-binding domain and are associated with the gain of the receptor function (Buchanan et al. 2001, Scher & Sawyers 2005). Overall, these observations suggest that AR signaling is important in the growth of PCa, as well as in the development of hormone resistance and relapse of the disease, and that if AR signaling and associated molecular events could be targeted by chemopreventive agents, this approach should help suppress PCa growth and progression.

Silibinin has been shown to decrease prostate specific antigen (PSA) protein expression in LNCaP cells together with cell growth inhibition via cell-cycle arrest (Zi & Agarwal 1999; Fig. 2). In a follow-up study, it was reported that silibinin and its crude form silymarin inhibit androgen-stimulated cell proliferation together with secretion of both PSA and human glandular kallikrein in LNCaP cells (Zhu et al. 2001). The study also showed that the transactivation activity of AR is diminished by both agents together with nuclear levels of AR; however, expression and steroid-binding ability of total AR were not affected (Zhu et al. 2001). Decursin is also reported recently to exert strong inhibitory effect on AR expression and activity in androgen-dependent PCa
cells (Jiang et al. 2006). Quercetin and selenium are reported to inhibit AR activity in LNCaP cells, which was accompanied by the reduction in cell proliferation (Morris et al. 2005). In another study, the inhibitory effect of quercetin on both expression and function of AR is reported in LNCaP PCa cells (Xing et al. 2001). Theaflavin-3,3',digallate and penta-O-galloyl-β-D-glucose have been shown to inhibit the activity of 5α-reductase, as well as the production of androgens (Lee et al. 2004a). Both these compounds also suppress the expression of AR and its activity in LNCaP cells leading to cell-growth inhibition. Genistein, an active component in soy diet, has also been shown to decrease AR expression and PSA secretion in LNCaP cells at physiological concentrations (Bektic et al. 2004). Whereas, the role of non-steroidal anti-inflammatory drugs (NSAIDs) has been well established in the chemoprevention of colon cancer, with regard to PCa, flufenamic acid, an NSAID, is found to transcriptionally inhibit the expression of AR and AR-promoter activity in LNCaP cells (Zhu et al. 2004). Ornithine decarboxylase (ODC) is an androgen-responsive gene and over-expressed in PCa (Mohan et al. 1999). It has been shown that green tea polyphenols inhibit testosterone-induced ODC activity, as well as anchorage-independent growth of LNCaP cells, and also inhibit ODC activity in rat prostate (Gupta et al. 1999). Together, these reports suggest that suppression of AR expression and activity by chemopreventive agents could have profound effect on PCa growth.

Receptor tyrosine kinase signaling

Epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor-type I (IGF-IR) have been identified as major tyrosine kinase membrane receptors, which are constitutively active in PCa (Lorenzo et al. 2003, Liao et al. 2005; Fig. 2). An increased production of growth factors to act as ligands and EGFR family receptors is the major contributing factor in the progression of PCa to the advanced and androgen-independent stages (Lorenzo et al. 2003). Many studies have shown the high levels of EGFR and transforming growth factor α (TGFα) in PCa that leads to the formation of an autocrine loop for a constitutively active mitogenic signaling in advanced human PCa cells (Mendelsohn & Baselga 2000, Lorenzo et al. 2003). With regard to their tissue levels, human PIN and primary and metastatic PCa show frequent expression of EGFR family receptors (Myers et al. 1994, Mendelsohn & Baselga 2000, Di Lorenzo et al. 2002). EGFR itself and other members in its family are usually activated by ligand binding and/or receptor homo/hetero-dimerization, and subsequently their intrinsic tyrosine kinase activity activity transmits both mitogenic and survival signals via Shc/Ras/Raf/MAPK and/or PI3K/Akt pathways (Whitmash et al. 1998, Fischer et al. 2003). Consistent with the observations regarding constitutively active EGFR signaling pathway, constitutive activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase1/2 (ERK1/2) and associated transcription factors is also observed and implicated in PCa progression to an androgen-independent and aggressive stage based on Gleason scores (Wasylyk et al. 1993, Gioeli et al. 1999). The role of EGFR activation in epithelial cancer growth is further demonstrated and established by the studies showing that inhibition of ligand binding to EGFR either by the antibodies, such as IMC-C225 and ABX-EGF or by the receptor tyrosine kinase inhibitors, such as Iressa and Tarceva, results in promising growth inhibitory outcomes at least in those epithelial cancers that express high levels of EGFR and its ligands (Mendelsohn & Baselga 2000, Lage et al. 2003). Together, these findings convincingly suggest that downregulation of EGFR signaling would be a vital strategy in the chemopreventive intervention of PCa.

To our knowledge, for the first time we showed that both silymarin and silibinin inhibit TGFα- and EGF-caused tyrosine phosphorylation of EGFR and its adaptor protein Shc in androgen-independent human PCa DU145 cells, which harbor constitutively active EGFR (Zi et al. 1998, Sharma et al. 2001). In the follow-up of detailed mechanistic investigations, silibinin inhibited ligand binding to EGFR and its internalization in to the cytoplasm, EGFR dimerization, ERK1/2 activation, as well as cell proliferation (Sharma et al. 2001). Silibinin also inhibited TGFα mRNA expression and decreased both secreted and cellular levels of TGFα in LNCaP and DU145 cells (Sharma et al. 2001). In a more recent study, we found that silibinin selectively exerts biological effects in 9L-EGFR cells (9L rat glioma cells lacking EGFR message were transfected with human EGFR for its overexpression) via inhibition of EGF-induced EGFR activation; the parental 9L glioma cells lacking EGFR did not respond to silibinin-caused inhibition of cell growth and proliferation (Hannay & Yu 2003, Qi et al. 2003). The findings of this study clearly suggest that growth inhibitory and apoptotic effects of chemopreventive agents, including silibinin could be achieved in PCa by targeting EGFR signaling. Consistent with this suggestion, other studies by us have shown that natural products, such as IP6 and GSE inhibit EGFR–ERK1/2 signaling together with growth and proliferation in
DU145 cells (Zi et al. 2000a, Tyagi et al. 2003a). Dietary genistein is also reported to inhibit EGFR and ERK1/2 expression and incidence of poorly differentiated prostatic adenocarcinomas in animal models (Wang et al. 2004). Other chemopreventive agents have also been shown to inhibit EGFR–ERK1/2 signaling in PCa (Bhatta & Agarwal 2001).

Similar to EGFR, IGF receptor (IGFR) signaling promotes growth and survival of PCa, and is constitutively active in the advanced stage of the disease, as well as closely linked to the PCa growth and progression in many studies (Yu & Rohan 2000, Liao et al. 2005; Fig. 2). Epidemiological studies in European and Western countries indicate a positive association between PCa risk and higher circulating levels of IGF-I and lower levels of IGF-binding protein (IGFBP)-3 (Chan et al. 1998, Chokkalingam et al. 2001). IGFs are mitogenic ligands and overexpressed in PCa, and their activity is firmly controlled by the presence of IGFBPs. IGF activates tyrosine kinase activity of IGFR and triggers downstream signaling either via PI3K/Akt and/or Ras/Raf/MAPK (Yu & Rohan 2000, Liao et al. 2005). IGFBPs sequester IGFs and reduce their availability to IGFR, and thereby downregulate IGFR activation (Grimberg & Cohen 2000). IGFs/IGFBP-3 plasma level is suggested as a potential predictor and end-point surrogate biomarker for PCa risk and progression (Harman et al. 2000, Chan et al. 2002). Interestingly, PSA has protease activity for IGFBP-3 (Cohen et al. 1994) that suggests a possible regulatory link between AR and IGFR signaling. Animal studies also suggest that IGFR signaling promotes growth and metastatic potential of PCa (DiGiovanni et al. 2000). Taken together, a well studied and established role of IGF-I-mediated mitogenic and survival signaling cascades in both PCa cells and animal models and their clinical relevance, convincingly suggest that it could be a promising target for chemopreventive agents.

Our studies show that silibinin downregulates IGFR-IR signaling in PCa and inhibits tumor cell growth, both in vitro and in vivo (Zi et al. 2000b, Singh et al. 2002b). The inhibitory effect of silibinin on IGFR-IR signaling was, in part, via an upregulation in both the message and protein levels of IGFBP-3, as well as its secreted levels. The use of IGFBP-3 antisense-oligonucleo-tide partially reversed the effect of silibinin on insulin receptor substrate-1 tyrosine phosphorylation and cell proliferation in human PCa PC3 cells (Zi et al. 2000b). In DU145 prostate tumor xenograft study, silibinin increased IGFBP-3 expression and secretion from tumor cells (Singh et al. 2002b, 2003b). Dietary silibinin is also observed to decrease IGF-I and increase IGFBP-3 levels in plasma of TRAMP mice (R P Singh, K Raina, MPollack & R Agarwal unpublished observations). Recently, apigenin is reported to inhibit IGF-IR signaling by decreasing IGF-I level with a concomitant increase in IGFBP-3 level in androgen-dependent PCa 22Rv1 cells, both in vitro and in vivo (Shukla et al. 2005). Green tea polyphenols have also been shown to increase IGFBP-3 and decrease IGF-I levels in TRAMP model, and the authors have claimed this to be the mechanisms of their efficacy in inhibiting prostate tumor growth in TRAMP model (Adhami et al. 2004). Genistein also downregulates IGFR-IR in TRAMP mice (Wang et al. 2004). Vitamin D analogs, including EB1089 have an anti-proliferative effect in PCa cells (Nickerson & Huynh 1999). In this study, EB1089-induced regression in prostate tumor has been shown to involve an alteration in the availability of IGF-I as a result of increased (15–25-fold) production of IGFBP-2 to IGFBP-5. The castration of rats also leads to upregulation of IGFBPs in the ventral prostate accompanied by its regression. These findings suggest that chemopreventive agents could potentially down-regulate IGFR-IR signaling by modulating the expression and interaction of IGF-I, IGFR-IR, and IGFBPs in both in vitro and in vivo PCa models, and that these effects of phytochemicals might be important contributing factors in their efficacy towards the inhibition of PCa growth and progression (Fig. 2).

**Signal transducers and activators of transcription (STATs) signaling**

STATs transduce mitogenic and survival signals of many growth factors and cytokines via tyrosine and serine phosphorylation by upstream kinases (Yu & Jove 2004). STAT signaling is under tight and transient regulation in normal cells, but becomes aberrant in many types of malignancies, including PCa (Ni et al. 2000, Yu & Jove 2004). Of the seven STAT proteins, STAT3 is persistently activated in PCa and most frequently in the hormone-resistant stage of the disease (Ni et al. 2000). The significance of STAT3 activation in PCa is further established by the studies showing that a disruption in its activation or expression results in growth inhibition and apoptotic death of PCa cells (Ni et al. 2000, Gao et al. 2005, Horinaga et al. 2005). Many STAT3-responsive elements have been identified in several genes that influence cell-cycle progression, such as cyclin D1 and angiogenesis, such as vascular endothelial growth factor (VEGF; Yu & Jove 2004). Interleukin (IL)-6 has been shown to mediate its oncogenic effect in LNCaP cells via Janus
kinases (usually JAK2) and STAT3 (Chen et al. 2000, Matsuda et al. 2001). Additionally, STATs are activated by cytokine receptors, G-protein coupled receptors or growth factor receptors having intrinsic tyrosine kinase activity, or by intracellular non-receptor tyrosine kinases (Yu & Jove 2004). Phosphorylation of STAT causes its dimerization, necessary for its nuclear translocation and transcriptional activity. STAT3 binds to human serum-inducible element (HSIE) and interferon-gamma activated site (GAS) sequences to mediate its anti-apoptotic and oncogenic effects (Yu & Jove 2004). Similar to AG490, a JAK2 tyrosine kinase inhibitor, antisense oligonucleotide for STAT3 inhibits STAT3 DNA-binding activity in DU145 cells and decreases the cell survival (Mora et al. 2002). Increased expression of STAT3 in tumors has been crucial in the processes evading immunological surveillance. Overall, these findings render STAT3 as a potential target for the intervention of PCa, including hormone-resistant phenotype of the disease.

Zyflamend, a herbal preparation and non-selective inhibitor of cyclooxygenase activity, is reported to inhibit STAT3 phosphorylation in LNCaP cells (Bemis et al. 2005). Studies with chemopreventive agents on STATs in PCa are limited. However, in other cancer cell types, such as multiple myeloma cells, curcumin is shown to inhibit constitutive and IL-6-induced STAT3 phosphorylation and nuclear translocation, as well as cell survival (Bharti et al. 2003). Curcumin has also been shown to inhibit interferon-α-induced STAT1 phosphorylation. Compared with AG490, curcumin is reported to be about 16 times faster and 10 times more effective inhibitor of STAT3 phosphorylation (Bharti et al. 2003). In other studies, curcumin is found to suppress JAK–STAT inflammatory signaling in brain microglia and T-cell leukemia (Kim et al. 2003, Rajasingh et al. 2006). Green tea polyphenols are shown to inhibit STAT1 phosphorylation and its DNA-binding activity in lung and colon epithelial cell lines for their anti-inflammatory activity (Watson et al. 2004). We have also observed the inhibitory effect of silibinin on STAT3 tyrosine phosphorylation in alveolar lung epithelial cancer A549 and human PCa DU145 cells (M Chittezhath, R P Singh & R Agarwal unpublished observations). Although, there is a considerable lack of studies assessing whether chemopreventive agents target STAT3 and/or inhibit its activation in PCa; a suggestion could be made for their modulatory effect on STAT signaling that most likely plays a critical role in their overall anti-PCa efficacy.

**β-catenin signaling**

β-catenin was first identified as a structural component in adherens junction formation participating with other proteins to regulate cell–cell adhesion. Later, cytoplasmic and nuclear β-catenin were described as potential effector of wingless-type MMTV integration site family (wnt) signaling associated with proliferation, differentiation, and metastasis, which is maintained at low levels in the absence of wnt stimulation (Chesire & Isaacs 2003). Adenomatous polyposis coli (APC) tumor suppressor and axin proteins form a complex with β-catenin and facilitate its N-terminal phosphorylation by glycogen synthase kinase-3β followed by its ubiquitination and proteosomal degradation (Davies et al. 2001). On the other side, enhanced β-catenin levels facilitate its nuclear translocation and interaction with DNA-binding transcription factors, more frequently with T-cell factor (TCF)/lymphoid enhancer factor family proteins (Chesire & Isaacs 2003, Yardy & Brewster 2005). The activated β-catenin upregulates genes (e.g., c-myc) by transactivation of TCF transcription factors, where it competes with their co-repressors for the binding and then recruits transactivating factors. In the nucleus, it also faces nuclear export by APC into the cytoplasm, as well as nuclear retention by TCF. Formation of PIN in transgenic mice expresses constitutively active β-catenin (Gounari et al. 2002). Alterations in APC or axins, or activating mutations in β-catenin itself have been observed to increase its nuclear activity in PCa, and therefore, indicates its oncogenic nature in tumor formation and progression (Gerstein et al. 2002, Yardy & Brewster 2005).

Chemopreventive agents, celecoxib, and α-difluoromethylornithine have been shown to restore β-catenin levels in the dorso-lateral prostate of TRAMP mice, and the authors have suggested these findings as an anti-metastatic effect of the agents (Gupta et al. 2000, 2004a). Since these studies did not examine the localization of β-catenin in the cytoplasmic membrane, cytoplasm and/or nucleus or its serine/threonine and tyrosine phosphorylation status, the authors’ claim is in contrast to the basic cell biology findings, because it is not clear how the increased level of β-catenin could have inhibited PCa growth and progression by these chemopreventive agents. Despite the advances in the understanding of basic mechanisms of wnt-β-catenin signaling, it remains mostly unexplored in PCa chemoprevention research. Therefore, more studies with known and new PCa chemopreventive agents are needed in this
direction to establish the role of β-catenin signaling in their efficacy against PCa.

**Toll-like receptor (TLR) signaling**

Chronic inflammation is an etiological event for many human cancers, including PCa. TLRs are among important mediators of inflammation as they recognize a broad range of microbial pathogens, including bacteria and viruses and interact with various adaptor proteins to activate different transcription factors, including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), and induce innate and adaptive immune response (Kawai & Akira 2006). Recent studies show that sequence variations in TLR4 gene (inherited polymorphisms) and TLR6–TLR1–TLR10 gene cluster are associated with PCa risk (Zheng et al. 2004, Chen et al. 2005, Sun et al. 2005), and that clinical PCa tissue harbor an enhanced expression of TLR1, TLR2, and TLR3 (Konig et al. 2004). Whereas, the functional role of TLR variants remains to be established in the growth and development of PCa, recently the role of TLR4 has been established in lung inflammation and tumorigenesis in animal models suggesting TLRs to be one of the potential targets for cancer chemoprevention (Bauer et al. 2005).

Many chemopreventive agents have been shown to inhibit downstream intermediate effector molecules of TLR signaling, such as MAPKs, AP-1, and NF-κB activation, as well as cytokine production in PCa; however, their modulatory effects on TLR activation remain to be studied. Helicobacter pylori infection stimulates TLR4 glycosylation, which initiates intracellular signaling in the infected host cell (Lee et al. 2004b). EGCG is shown to completely block TLR4 glycosylation and activation resulting in an inactivation of ERK1/2 and NF-κB and a decreased synthesis of the proinflammatory mediator hydroxyeicosatetraenoic acid in gastric mucosa (Lee et al. 2004b). Since many intermediate signaling molecules are common to other membrane receptor signaling, genetic manipulations, such as knockout/knockdown need to be employed to define the effect of chemopreventive agents specifically on TLR signaling in PCa.

**Cell-cycle regulatory targets**

**Cyclin-dependent kinases (CDKs) and cyclins**

Deregulated cell-cycle progression is a fundamental biological phenomenon underlying the growth and development of cancer. Cell-cycle progression is regulated by the activity of CDKs (serine/threonine kinases) in non-covalent association with their regulatory subunits, cyclins, and CDK inhibitors (CDKIs; Sherr 2000, Vermeulen et al. 2003). Activation of different CDKs is specific to the distinct phases of the cell cycle, for example, CDK4 and CDK6 with D-type cyclins are activated for early and mid-G1-phase progression, whereas CDK2–cyclin E for late-G1 and G1–S transition, CDK2–cyclin A for S-phase progression, and Cdc2 (or CDK1)–cyclin B for G2–M phase transition (Grana & Reddy 1995, Sherr & Roberts 1999, Sherr 2000, Senderwicz & Sausville 2000). Further, Cdc25 tyrosine phosphatases regulate G2–M checkpoint transition (Nilsson & Hoffmann 2000), where Cdc25 dephosphorylates Cdc2 facilitating a complex formation with cyclin B to drive the cell through G2–M checkpoint transition. In genotoxic stress, ataxia telangiectasia-mutated (ATM)/ataxia telangiectasia and rad3 (ATR)-related kinase–checkpoint kinase (Chk)1/2 cascade activation causes inhibitory phosphorylation of Cdc25 to halt the cell-cycle progression at G2–M transition (Kawabe 2004). Various genetic and epigenetic changes or oncogenic alterations enhance Cdc2 kinase activity in many cancers, including PCa (Fu et al. 2004a, Kawabe 2004). Additional studies also show the overexpression/activation of cyclin/CDK and their activating phosphatases (Cdc25A/B) in many cancer cells, including PCa (Grana & Reddy 1995, Fu et al. 2004a), and that many prostate tumors carry mutations, which deregulate at least one of the CDKs; most frequently CDK2, CDK4, CDK6, or CDK1. Moreover, neoplastic mitogenic signaling is linked to the activation of CDK–cyclin complex for uncontrolled cell proliferation and survival (Fig. 2). Taken together, these studies suggest that a correction in the regulation of unchecked cell-cycle progression, or alternatively a cell-cycle arrest, could be an effective strategy to control the growth and proliferation of cancer cells and to induce their apoptotic death. As summarized next, various chemopreventive agents have shown promising outcomes in these directions.

Silibinin has been reported to induce G1 arrest in human and rat PCa cells together with an inhibition in the kinase activity of CDK4, CDK6, and CDK2, which are known to regulate G1 phase of the cell cycle (reviewed recently by Singh & Agarwal 2004). The observed inhibitory effect of silibinin on the kinase activity of various CDKs was in part due to a strong decrease in the protein levels of both CDKs and cyclins, and was evidenced in both androgen-independent (DU145) and androgen-dependent (LNCaP) human PCa cells (Zi et al. 1998, Zi & Agarwal 1999). Similar observations have also been reported.
recently with apigenin in DU145 cells (Shukla & Gupta 2004a,b), and its analogs, such as acacetin and linarin DU145, PC3, and LNCaP cells (Singh et al. 2005a). We have also reported recently that the G1 arrest-inducing effect of decursin in human PCa cells is via a decrease in the levels of CDKs and cyclins and associated kinase activity (Yim et al. 2005). Similarly, as reviewed recently by us, EGCG, genistein, and quercetin also induce G1 arrest in various PCa cells by modulating CDK–cyclin activity (Agarwal 2000, Singh et al. 2002a). Together, these studies suggest that chemopreventive agents potentially decrease the protein levels of CDKs and/or cyclins that in part are responsible for an inhibition in the kinase activity of CDKs causing a halt in deregulated cell-cycle progression of PCa cells (Fig. 2).

More recently, silibinin has also been shown to decrease Cdc2 and cyclin B1 expression and associated kinase activity leading to a G2–M arrest in PCa PC3 cells (Deep et al. 2006). In this study, silibinin also decreased Cdc25B and Cdc25C protein levels, but increased Cdc25C(Ser216) phosphorylation leading to its enhanced binding with 14-3-3β and cytoplasmic retention in an inactive form. Further, silibinin enhanced Chk2 phosphorylation at Thr68/Ser19 site, which was responsible for Cdc25C phosphorylation as confirmed by the use of Chk2 siRNA (Deep et al. 2006). This study, for the first time, showed that silibinin causes inhibitory activation of Chk2–Cdc25C–Cdc2/cyclin B1 cascade leading to G2–M arrest and associated apoptosis in human PCa cells. Other chemopreventive agents have also been shown to induce G2–M arrest in PCa cells by inhibiting Cdc2–cyclin B1 activity (Singh et al. 2002a).

**CDK inhibitors**

CDKIs (such as Cip1/p21, Kip1/p27, and Kip2/p57) physically interact with CDK–cyclin complexes to negatively regulate CDK activity and cell-cycle progression (Sherr & Roberts 1999, Dai & Grant 2003). Whereas, Cip1/p21 is involved in all phases of the cell cycle and therefore known as a universal inhibitor of various CDKs’ kinase activity, Kip1/p27 usually regulates cell-cycle progression through G1–S checkpoint transition (Xiong et al. 1993, Toyoshima & Hunter 1994, Dai & Grant 2003). These CDKIs bind at ATP-binding sites in cyclin–CDK complex and inhibit CDK activity. Cip1/p21 is regulated by many agents in p53-dependent or -independent manner, and is altered at both transcriptional and post-transcriptional levels (Zi et al. 1998, Hartel & Tyner 2002). The down-modulation of CDKIs is facilitated by ubiquitination and proteosomal degradation involving Skp2 (Bloom & Pagano 2003). Another family of CDKIs includes INK4 (INK4b/p15, INK4a/p16, INK4c/p18, and INK4d/p19); the members in this family specifically bind to and inhibit CDK4 and CDK6 activity (Hirai et al. 1995, Ortega et al. 2002). CDKI/INK genes are often mutated and/or have very low levels of expression in cancer cells that lead to a loss in their control on cell-cycle progression (Tsihlias et al. 1999, Ortega et al. 2002). Interestingly, mitogenic signals also decrease the levels of CDKIs, and anti-mitogenic signals induce their expression that inhibits cell proliferation; this approach has provided a potential strategy for cancer control (Liu et al. 1996). The importance of INK has been shown in CDK4 knockout mice, in which normal CDK4 gene was replaced by a CDK4 R24C (Arg24 replaced with cysteine making it insensitive to INK4) mutant (Sotillo et al. 2001). These mice develop a wide spectrum of spontaneous tumors and are highly susceptible to carcinogens. Recent reports also suggest the prognostic significance of these CDKIs in PCa (Tsihlias et al. 1999). Overall, these studies convincingly suggest that chemopreventive agents targeting CDKIs for their upregulation could be a valuable strategy against PCa growth and progression (Fig. 2).

Studies by us have shown that silibinin induces protein expression of Cip1/p21 and Kip1/p27 followed by their increased interaction with CDK2, CDK4, and CDK6, and subsequent inhibition of CDKs’ activity in human PCa cells (Zi et al. 1998, Zi & Agarwal 1999, Deep et al. 2006). Other agents, such as IP6 also increases Kip1/p27 and Cip1/p21 protein levels in a dose-dependent but p53-independent manner, and induces G1 arrest in human PCa cells (Singh et al. 2003a, Agarwal et al. 2004a). IP6-induced increase in Kip1/p27 and Cip1/p21 was also associated with their increased binding with CDKs and cyclins, and subsequent decrease in the kinase activity of G1-phase CDKs. In our ongoing study with siRNA for Kip1/p27 and Cip1/p21, these functional genes were observed decisive in causing both silibinin and IP6-induced G1 arrest in DU145 cells (R P Singh, S Roy & R Agarwal unpublished observations). Grape seed extract (GSE) has also been shown to induce Kip1/p27 and Cip1/p21 protein levels and associated G1 arrest in DU145 cells (Agarwal et al. 2000). Yet again, decursin-caused G1 arrest in DU145 cells has been associated with an increased expression of Cip1/p21 and Kip1/p27 protein levels (Yim et al. 2005). Apigenin has also been shown to induce Cip1/p21, Kip1/p27, INK4a/p16, and INK4c/p18 protein levels and cell-cycle arrest in DU145 cells (Shukla & Gupta 2004a,b). Similarly, EGCG is shown to induce G1 arrest in DU145 PCa cells
via induction of INK4c/p18 and INK4a/p16 with a concomitant decrease in the CDKs’ activity and has been reviewed recently (Singh et al. 2002a). Since increased rate of cancer progression, aggressive phenotype, and decreased response to therapy are linked to the loss and/or low expression of CDKIs in PCa (Chau & Wang 2003), these findings suggest that upregulation of CDKIs by chemopreventive agents could inhibit PCa growth and progression.

**Retinoblastoma protein and E2F**

Retinoblastoma (Rb/p110) and Rb-family proteins, such as Rb/p107 and Rb2/p130 have been reported as important players in cell-cycle regulation and are mostly inactivated in cancer cells, including PCa (Paggi et al. 1996, Chau & Wang 2003). These nuclear phosphoproteins are phosphorylated by CDKs (CDK4/6–cyclin D1 and CDK2–cyclin E complexes) and subsequently become unable to sequester E2F family of transcription factors (Chau & Wang 2003, Seville et al. 2005). Thus free E2Fs, in response to mitogenic signal, induce gene transcription required for the cell-cycle progression (Chau & Wang 2003). Conversely, hypophosphorylated Rb and Rb-related proteins sequester E2Fs and inhibit cell proliferation. Rb-family genes are critically targeted for inactivation by transforming oncoproteins, and Rb is mutated in many cancers, including PCa (Chau & Wang 2003, Seville et al. 2005). High levels of E2Fs have also been observed in neoplastic cells. It is also important to emphasize here that E2Fs are downstream targets for the tumor suppressor p53, as well as Rb proteins (Slansky & Farnham 1996), and that most of the advanced PCa tissue samples and established prostate carcinoma cells show increased E2F transcriptional activity as a consequence of loss of p53 and/or Rb (Chau & Wang 2003). Rb/p107 and Rb2/p130 co-operate to regulate cell-cycle progression through G1 phase with a common goal of G1–S checkpoint regulation (Sage et al. 2000). These studies suggest that upregulation of Rb-family proteins, as well as their increased hypophosphorylation by chemopreventive agents could check the deregulated cell-cycle progression in PCa (Fig. 2).

Consistent with the above suggestion, studies by us have shown that silibinin increases hypophosphorylated levels of Rb and Rb-related proteins together with their increased binding with E2Fs, and that this effect of silibinin was associated with growth inhibition via G1 arrest in human PCa cells (Tyagi et al. 2002a,b). Consistent with a decrease in CDK–cyclin kinase activity in LNCaP cells, silibinin increased hypophosphorylated levels of Rb/p110 with a specific decrease in Rb/p110 phosphorylation at Ser780, Ser795, Ser807/811, and Ser249/Thr252 sites (Tyagi et al. 2002a). Silibinin also caused a moderate to strong decrease in E2F1, E2F2, and E2F3 levels in LNCaP cells (Tyagi et al. 2002a), and E2F4 and E2F5 levels in DU145 cells (Tyagi et al. 2002b). Overall, these effects of silibinin were suggestive of suppression in E2F transcriptional activity leading to a G1 arrest in PCa cell-cycle progression. We have also observed that IP6 modulates Rb-related protein–E2F complex in causing G1 arrest in human PCa cells. IP6 increases hypophosphorylated levels of Rb/p110 in LNCaP cells, and Rb/p107 and Rb2/p130 in DU145 cells (Singh et al. 2003a,b, Agarwal et al. 2004a), together with an increase in pRb/p107 and pRb2/p130 interaction with E2F4 and a decrease in the level of transcriptionally active-free E2Fs leading to growth inhibition of PCa cells via G1 arrest (Singh et al. 2003a). The decreased phosphorylation of Rb family proteins is a consequence of the inhibitory effect of IP6 on CDK–cyclin kinase activity (Singh et al. 2003b, Agarwal et al. 2004a). Recently, a novel flavonoid licochalcone, isolated from the roots of licorice, is reported to inhibit Rb phosphorylation at Ser780 together with a decrease in the levels of different E2Fs in PC3 cells and this effect was associated with G2–M cell-cycle arrest (Fu et al. 2004b). Equiguard, a polyherbal supplement, inhibits Rb phosphorylation, as well as exhibits anti-tumor activity against LNCaP cells (Lu et al. 2004). Indole-3-carbinol also decreases hyperphosphorylated levels of Rb in PCa cells (Sarkar & Li 2004). Overall, these reports suggest potential anti-cancer activity of chemopreventive agents via targeting Rb–E2F complexes most likely via their modulatory effects on CDK–cyclin–CDKI axis in PCa.

**Telomerase**

The telomerase (a reverse transcriptase enzyme) activity is essential to maintain a specialized heterochromatin structure, a tandem repeats of the hexanucleotide sequence with a terminal 3' G-rich overhang called telomere, which protects the ends of chromosomes (Neumann & Reddel 2002, Biroccio & Leonetti 2004). Telomeres shorten during each cell division, and normal cells stop replicating once telomeres become critically short; however, almost all human tumor cells acquire telomerase activity to overcome this limitation (Kim et al. 1994, Mathon & Lloyd 2001, Orlando et al. 2001). Telomerase activity is absent in normal prostate; however, primary prostate tumors show elevated telomerase activity that is also correlated with their
aggressiveness (Lin et al. 1997, Kim & Hruszkewycz 2001, Orlando et al. 2001). Furthermore, androgen depletion has been shown to activate telomerase in the prostate of monkeys (Ravindranath et al. 2001), and therefore, could contribute to PCa progression during anti-androgen therapy. These studies suggest that telomerase activity could be another potential target for chemopreventive agents in their efficacy against PCa growth and progression (Fig. 2).

By using telomeric repeat amplification protocol assay, it has been shown that androgen-sensitive LNCaP cells possess DHT-dependent telomerase activity, and silibinin has been shown to decrease basal, as well as DHT-induced mRNA expression of telomerase catalytic subunit (Thelen et al. 2004). It has been suggested that the inhibitory effect of silibinin on DHT-caused increase in telomerase activity via AR possibly involves the downregulation of telomerase and other genes, including PSA. Though not in PCa, EGCG has been reported to downregulate telomerase activity in human breast carcinoma MCF-7 cells, which has been associated with the suppression of cell viability (Mittal et al. 2004). Nevertheless, more studies are needed to establish telomerase as a potential target for chemopreventive agents in their efficacy against PCa.

Cell survival/apoptotic targets

NF-κB pathway

NF-κB has been implicated in the growth and survival of many types of cancer cells, including PCa (Karin et al. 2002). NF-κB is a family of homo- or heterodimeric transcription factors, including p50, p52, p65, RelB, and c-Rel (Schmitz & Baeuerle 1995, Karin et al. 2002). NF-κB is sequestered by inhibitory-κB (IκB) proteins in cytoplasm, which are phosphorylated at serine sites by IκB kinases (IKKs) followed by their ubiquitination and degradation in response to anti-apoptotic/survival signals (Baeuerle 1998, Karin et al. 2002). Thus, free NF-κB translocates to the nucleus and binds to a common DNA sequence motif, known as κB site, in a broad spectrum of genes that include inflammatory cytokines, chemokines, cell adhesion molecules, and growth and survival genes (Giuliani et al. 2001, Karin et al. 2002). ERK1/2 and Akt pathways are also known to activate NF-κB (Agarwal et al. 2005, Hsiung et al. 2005, Jeong et al. 2005). Both constitutive and induced activation of NF-κB play a crucial role in the development of chemotherapy resistance in various cancer cells, including PCa (Flynn et al. 2003). NF-κB is constitutively active in androgen-independent PCa, where it causes the activation of many genes, including those involved in apoptosis resistance, angiogenesis, invasion, and metastasis (Sumimoto et al. 1999, Lindholm et al. 2000, Karin et al. 2002, Suh & Rabson 2004). Together, these studies suggest that targeting NF-κB activation by chemopreventive agents could be a logical strategy to control PCa growth, tumor angiogenesis, metastasis, and chemoresistance.

Our studies have shown that silibinin inhibits IKKα kinase activity accompanied by an inhibition in IκBα phosphorylation and NF-κB transcriptional activity in androgen-independent PCa DU145 cells (Dhanalakshmi et al. 2002). Silibinin also showed a decrease in nuclear translocation of p65 and p50 with a concomitant increase in their cytoplasmic levels. In this study, we also found that silibinin strongly increases the sensitivity of DU145 cells to otherwise resistant TNFα-induced apoptosis, and that this effect of silibinin was via an inhibition of TNFα-induced NF-κB activation (Dhanalakshmi et al. 2002). The inhibitory effect of silibinin on NF-κB signaling could be an important mechanism of its anti-tumor efficacy observed in many studies. IP6 has also been shown to inhibit NF-κB activation and cell survival in human PCa DU145 cells (Agarwal et al. 2003). Similarly, indole-3-carbinol is shown to inhibit NF-κB activation in advanced human PCa cells and sensitizes them for chemotherapy (Sarkar & Li 2004). It has been reported recently that curcumin and phenethyl isothiocyanate inhibit the growth of PC3 prostate tumor xenografts in nude mice, and that they inhibit NF-κB activation; the effect of these two agents was stronger when combined together than each agent alone (Khor et al. 2006). Many other chemopreventive agents, such as genistein, EGCG, and selenium compounds have been shown to inhibit NF-κB activation in different PCa cells as one of their mechanisms of chemopreventive efficacy (Davis et al. 1999, Gasparian et al. 2002, Gupta et al. 2004b). Overall, these studies clearly suggest that NF-κB signaling is an important target for chemopreventive agents to check the growth, survival, and drug resistance in PCa, and that the effective inhibitors of this pathway have potential application in clinic.

ATM kinase and cell-cycle Chk signaling

Genotoxic stress is known to activate ATM and ATR serine/threonine protein kinases, which phosphorylate a histone variant H2AX(Ser139) marking an early event in DNA damage/apoptosis and associated cell-cycle arrest (Yang et al. 2003). These kinases also phosphorylate Chk1 and Chk2 that subsequently cause
inhibitory phosphorylation of Cdc25C, which otherwise dephosphorylates Cdc2 making it active for cell-cycle progression; phosphorylated Cdc2 is inactive and causes cell-cycle arrest in S or G2–M phase (Tenzer & Pruschy 2003). The cell-cycle arrest is accompanied by DNA repair, if the damage is moderate, or an induction of apoptosis, if the damage is excessive and could not be repaired (Tenzer & Pruschy 2003, Zhou et al. 2003). Recently, it has been observed that downregulation of ATM by antisense-ATM oligonucleotide sensitizes androgen-dependent (LNCaP and CWR22-Rv1) and androgen-independent (PC3 and DU145) human PCa cells to radiation-induced apoptosis (Truman et al. 2005). In another study, ATM knockdown by siRNA in p53-defective PC3 cells has been shown to increase the sensitivity of the cells to doxorubicin-induced apoptosis (Mukhopadhyay et al. 2005). Furthermore, a higher expression of ATM protein has been observed in the higher grade PCa tissues as compared with that of normal prostate (Angele et al. 2004); however, it has also been reported that defective DNA-strand break repair happens in PCa cells (LNCaP, DU145, and PC3) as compared with normal prostate epithelial cells (Fan et al. 2004). The aberrant DNA repair could be a possible mechanism for acquiring genetic instability during PCa progression or radiotherapy. Therefore, it is plausible that inhibiting DNA repair by blocking ATM signaling or its increased activation with the lack of normal DNA repair might sensitize PCa cells for both cell-cycle arrest and apoptosis.

Recently, we observed that silibinin activates ATM–Chk2 pathway leading to H2AX(Ser139) phosphorylation together with a G2–M arrest and apoptosis in PC3 cells (Deep et al. 2006). In this study, silibinin-caused ATM(Ser1981) phosphorylation resulted in cell-cycle arrest and apoptosis through Chk2 activation, as Chk2 siRNA abrogated the biological responses of silibinin (Deep et al. 2006). Other studies have shown that natural chemopreventive agents induce ATM(Ser1981) phosphorylation in many types of cancer cells, including PCa. For example, genistein causes ATM-dependent activation of Chk2 and G2–M arrest in liver carcinoma HepG2 cells (Chang et al. 2004). Apigenin, luteolin, and kaempferol are also reported to activate ATM/ATR kinases for G2–M cell-cycle arrest (O’Prey et al. 2003). Overall, these studies suggest that ATM signaling could be potentially targeted by chemopreventive agents to induce G2–M arrest and apoptosis in PCa cells. Since these findings are against those showing that downregulation of ATM decreases the survival of PCa cells, more studies are needed to further investigate these discrepancies. Nonetheless, studies are also needed to identify the in vivo significance of the modulatory effect of chemopreventive agents on ATM signaling in PCa.

**Bcl-2 family proteins**

The growth of malignant cells, including PCa is almost always accompanied by the loss of apoptotic response, and therefore, an induction of apoptosis in cancer cells is long being used as a strategy to control PCa growth and progression. Further, the agents with both growth inhibitory and apoptotic efficacy could be more effective in both prevention and intervention of PCa. Activation of the caspase cascade is commonly observed in the execution of apoptosis (Ho & Hawkins 2005, Watson & Fitzpatrick 2005). Caspase (a family of cysteine proteases) ultimately cleaves many substrates proteins, including poly-(ADP-ribose) polymerase (PARP) that marks the induction of apoptotic cell death (Ho & Hawkins 2005). In mitochondrial apoptosis, the damage of mitochondrial integrity followed by the release of cytochrome c forming a complex with Apaf-1 and pro-caspase 9 initiates the activation of a caspase-mediated apoptotic cascade (Reed 2002, Ho & Hawkins 2005, Watson & Fitzpatrick 2005). Bcl-2 family members, consisting of both anti-apoptotic (such as Bcl-2 and Mcl-1) and pro-apoptotic (such as Bax and Bad) molecules, play major roles in regulating the redox state of mitochondria (Reed 2002, Catz & Johnson 2003). The balance and interaction between these proteins determine the survival or apoptotic fate of the cell (Catz & Johnson 2003). In cancer cells, including PCa, the equilibrium in terms of the content and interaction of these proteins is shifted away from the pro-apoptotic end towards the survival end and, therefore, cells become resistant to apoptotic death (Catz & Johnson 2003). Several studies have shown an overexpression of Bcl-2 in about 30% of the PCa cases, which was also correlated with the advanced stage of the disease and poor prognosis (Raffo et al. 1995, Moul 1999, McCarty 2004). Moreover, advanced PCa also develops apoptosis resistance towards the commonly used androgen ablation therapy (Raffo et al. 1995, Moul 1999). Antisense oligonucleotides or siRNA for Bcl-2 or Bcl-xL, as well as a forced expression of Bax have been shown to induce apoptosis in PCa cells (Lin et al. 2005, Yamanaka et al. 2005). Thus, chemopreventive agents that shift the equilibrium from anti-apoptotic to pro-apoptotic Bcl-2 family members leading to apoptosis induction could be promising in controlling the growth and survival of PCa cells.

Silibinin has been shown to induce apoptosis and inhibit DU145 tumor xenograft growth (Singh et al.
2003b); however, its effect on Bcl-2 family members as not yet been investigated. The growth inhibitory effect of IP6 has been shown to be associated with an induction of apoptotic death in human LNCaP and DU145, as well as in mouse TRAMP-C1 PCa cells as reviewed by us recently (Singh & Agarwal 2005). IP6 also causes in vivo apoptotic death of PCa cells in DU145 tumor xenograft (Singh et al. 2004a). In mechanistic studies, IP6 was found to activate caspases and to cause PARP cleavage in human PCa cells (Singh et al. 2003a). In mouse PCa cells, however, the use of a pan-caspase inhibitor shows the involvement of both caspase-dependent and -independent mechanisms in IP6-induced apoptotic cell death (Sharma et al. 2003). IP6 also increases the ratio of Bax:Bcl-2 in LNCaP cells, which was associated with increased apoptosis (Agarwal et al. 2004a). GSE also induces apoptotic death through dissipation of mitochondrial membrane potential and cytochrome c release causing the activation of caspase cascade in DU145 cells (Agarwal et al. 2002). In this study, GSE also showed an increase in the cleavage of pro-caspases 3 and 9 together with an increase in their protease activity. The use of caspase inhibitors showed that GSE-induced apoptosis was mostly via caspase activation. Recently, pomegranate fruit extract has been shown to induce pro-apoptotic Bax and Bak levels together with a decrease in the levels of anti-apoptotic proteins, namely Bcl-2 and Bcl-xL in PC3 cells; these effects were associated with a decrease in cell survival (Malik et al. 2005). Selenium compounds have been shown to decrease the ratio of Bcl-2:Bax in human PCa-derived cells (Husbeck et al. 2006). Similarly, EGCG has also been shown to cause a change in Bcl-2:Bax ratio in LNCaP cells that favors apoptosis (Hastak et al. 2003). Diallyl trisulfide and phenethyl isothiocyanate from cruciferous vegetables have been shown to induce Bad or Bak, and decrease Mcl-1 or Bcl-xL in PCa cells together with an apoptosis induction (Xiao & Singh 2005, Xiao et al. 2005). Recently, many other chemopreventive agents, including quercetin (Vijayababu et al. 2005), EGCG (Hastak et al. 2005), sulforaphane (from cruciferous vegetables; Choi & Singh 2005), β-lapachone (from a South American tree Tabebuia avellanedae; Lee et al. 2005), retinoic acid (Huynh et al. 2006), neem (Azadirachta indica) leaf extract (Kumar et al. 2005), and guggulsterone (from a medicinal plant, Commiphora mukul; Singh et al. 2005c) have been shown to increase the ratio of pro-apoptotic vs anti-apoptotic Bcl-2 family proteins and induce apoptosis in different PCa cells. Overall, these studies suggest that chemopreventive agents have potential to target Bcl-2 family proteins, though these observations need to be further explored in pre-clinical and clinical prostate tumors.

**Inhibitor of apoptosis protein (IAP)**

IAP family is known to have pleiotropic functions, including regulation of cell-cycle progression and inhibition of extrinsic and intrinsic pathways of cell death (LaCasse et al. 1998, Altieri 2003). Survivin, the smallest (16.5 kDa) protein in the family, shows very rare expression in normal tissues excluding embryonic tissues and those with a high rate of cellular turnover, such as colonic mucosa; however, it is overexpressed in many types of cancers, including PCa (LaCasse et al. 1998, Altieri 2003, Krajewska et al. 2003, Kishi et al. 2004). Survivin expression is also associated with biologically aggressive phenotype and drug resistance in prostate tumors (Kishi et al. 2004, Shariat et al. 2004). Survivin directly interacts with caspase-3 and caspase-7 leading to an inhibition in their protease activity and subsequent apoptosis. Downregulation of survivin expression by anti-sense has been shown to potentiate apoptosis induction by the chemotherapeutic agents, such as docetaxel and etoposide in DU145 cells (Hayashi et al. 2005). Together, these studies suggest that a differential expression of survivin could make it a selective target for chemopreventive agents in terms of inducing apoptotic death only in cancer cells.

In a preliminary study, silibinin is observed to moderately decrease survivin protein expression in PCa cells (unpublished data). Our completed studies in other cancer cell types and human endothelial cells show that silibinin-caused apoptosis is associated with a decrease in survivin protein expression concomitant with an increase in caspase activity (Tyagi et al. 2003b, Mallikarjuna et al. 2004, Singh et al. 2005b). Genistein is shown to inhibit survivin expression in human PCa LNCaP cells (Suzuki et al. 2002). The combination of vitamins C and D is reported to inhibit survivin mRNA expression in DU145 cells (Gunawardena et al. 2004). However, the lack of sufficient studies with chemopreventive agents in terms of their effect on the modulation of survivin levels in PCa, suggests that more investigations are needed to define survivin’s role in apoptotic cell death in PCa by these agents.

**Angiogenic and metastatic targets**

**Tumor angiogenesis and angiogenic factors**

Angiogenesis is a prerequisite for the growth of solid tumors and their metastasis. Tumors remain avascular and latent for years; however, tumor growth can be initiated by neo-angiogenesis (Bergers & Benjamin 2002). In this study, GSE also showed an increase in Bcl-2:Bax ratio in human PCa-derived cells (Singh et al. 2002). The combination of anti-angiogenic factors and chemopreventive agents showed that silibinin-caused apoptosis is associated with a decrease in survivin protein expression concomitant with an increase in caspase activity (Tyagi et al. 2003b, Mallikarjuna et al. 2004, Singh et al. 2005b). Genistein is shown to inhibit survivin expression in human PCa LNCaP cells (Suzuki et al. 2002). The combination of vitamins C and D is reported to inhibit survivin mRNA expression in DU145 cells (Gunawardena et al. 2004). However, the lack of sufficient studies with chemopreventive agents in terms of their effect on the modulation of survivin levels in PCa, suggests that more investigations are needed to define survivin’s role in apoptotic cell death in PCa by these agents.
Tumor vasculature is also recognized as a prognostic marker and predictor of malignant potential of PCa (Bostwick & Iczkowski 1998, Bono et al. 2002, Turner et al. 2003). Further, vascular endothelial cells are less likely to acquire therapeutic resistance and are directly exposed to the physiologically achievable agents, including chemopreventive agents (Bergers & Benjamin 2003). The critical dependence of solid tumors on angiogenesis suggests that blocking angiogenesis could be a potential strategy in controlling PCa growth. The potential anti-angiogenic strategies targeted by chemopreventive agents could include the blocking of production and release of angiogenic factors, increase in anti-angiogenic factors, and inhibition of endothelial cell proliferation, migration and survival, and disruption of the process of microvessel formation (Fig. 3). Chemopreventive agents have shown pleiotropic anti-angiogenic effects in many studies as reviewed by us recently (Singh & Agarwal 2003). It is important to highlight here that many anti-angiogenic agents, including phytochemicals, antibodies, and synthetic peptides are already in clinical trials (Kerbel 2000).

Tumor cells synthesize and secrete angiogenic factors, and via paracrine regulation of endothelial cells, initiate neo-angiogenesis from pre-existing blood vessels (Ferrara 2002, Bergers & Benjamin 2003; Fig. 3). VEGF, basic fibroblast growth factor (bFGF), and IGF-I secreted from tumor cells are known to stimulate tumor angiogenesis (Ferrara 2002, Bergers & Benjamin 2003). COX-2, nitric oxide synthase (NOS), and IL-8 are among the inflammatory angiogenic molecules produced by tumor cells that influence neo-angiogenesis (Chiarugi et al. 1998, Bergers & Benjamin 2003). In tumor cells, the expression of these angiogenic molecules is favored by the constitutively active mitogenic and survival signaling, including those mediated via EGF receptor, IGFR, and platelet-derived growth factor receptor, ERK1/2 and Akt (Ferrara 2002, Singh & Agarwal 2003). Since most of these signaling pathways
are inhibited by various chemopreventive agents in PCa, a decreased expression of angiogenic factors was also anticipated as reported in many studies.

Silibinin-caused inhibition of mitogenic signaling has been shown to be associated with a decrease in both expression and secretion of VEGF in human PCa cells, as reviewed by us recently (Singh & Agarwal 2004). EGCG is also shown to inhibit receptor tyrosine kinase signaling causing a decrease in both expression and secretion of VEGF and bFGF in many cancer cell types (Sartippour et al. 2002, Lee et al. 2004a,b, Rodriguez et al. 2006). Furthermore, silibinin, EGCG, genistein, resveratrol, and several other agents have been shown to decrease COX-2 and/or iNOS expression in different cancer cells, suggesting them as the putative targets for the suppression of tumor angiogenesis. These chemopreventive agents also inhibit NF-κB activity that is known to mediate angiogenesis, invasion, and metastasis in PCa as discussed earlier. These facts suggest that the inhibition of oncogenic signaling in PCa by chemopreventive agents could potentially decrease the production of angiogenic factors by tumor cells and associated angiogenesis.

**VEGF receptor signaling**

VEGF is the most important mitogenic and survival factor for vascular endothelial cells (Ferrara 2002, Turner et al. 2003). VEGF interacts with high affinity to both fms-like tyrosine kinase-1 (FLT-1) and fetal liver kinase-1 (Flk-1/KDR) receptors, which are primarily expressed by vascular endothelial cells (Ferrara 2002). However, these receptors are also expressed in many PCa cells, suggesting a possible autocrine loop for VEGF–VEGF receptor signaling (Turner et al. 2003). The binding of VEGF to its receptors stimulates receptor dimerization and activation leading to the propagation of signaling cascade for enhanced proliferation and survival of endothelial cells (Ferrara 2002, Turner et al. 2003). Therefore, disrupting VEGF receptor signaling could be an attractive target for chemopreventive agents to check the growth and development of PCa, given that angiogenesis is a prerequisite for tumor growth and metastasis (Fig. 3).

Our studies show that silibinin strongly inhibits growth and survival of endothelial cells (Singh et al. 2005b). Silibinin is also observed to inhibit VEGF- and IGF-I-induced cell proliferation and survival in human endothelial cells (R P Singh & R Agarwal unpublished observations). Similarly, GSE also inhibits growth and survival of endothelial cells (Agarwal et al. 2004b). Genistein is shown to inhibit VEGF-induced tyrosine phosphorylation of receptor kinases in endothelial cells (Guo et al. 1995). EGCG, catechin-3 gallate, and epicatechin-3 gallate have been shown to inhibit VEGF-induced tyrosine phosphorylation of Flk-1/KDR and suppress in vitro angiogenesis in endothelial cells (Lamy et al. 2002). Moreover, an endogenous anti-angiogenic protein, endostatin is shown to block VEGF-induced activation of Flk-1/KDR, ERK, and p38MAPK (Kim et al. 2002). Costunolide, a sesquiterpene lactone isolated from *Saussurea lappa* has been shown to inhibit Flk-1/KDR signaling in human vascular endothelial cells (HUVEC) together with a suppression in VEGF-induced endothelial cell proliferation and neo-vascularization of mouse cornea (Jeong et al. 2002). IP6 also inhibits bFGF-induced in vitro angiogenesis and reduces VEGF expression (Vucenik et al. 2004). These reports suggest that targeting VEGF receptor signaling by chemopreventive agents could be a potential anti-angiogenic mechanism to halt tumor angiogenesis in PCa.

**Hypoxia-inducible factor-1α**

Hypoxia is known to be associated with an increased tumor metastasis and poor survival in cancer patients (Zhong et al. 1999, Harris 2002). Hypoxia activates angiogenesis and stimulates vascular remodeling (Harris 2002). Hypoxia inducible transcription factor (HIF)-1 is comprised of two subunits, HIF-1α and constitutively expressed HIF-1β, and controls the expression of several genes regulated by hypoxia (Harris 2002). Tumor suppressor Von Hippel-Lindeau (VHL) usually binds to and causes proteosomal degradation of HIF-1α (Pugh & Ratcliffe 2003). Hypoxia or a mutation in VHL gene increases HIF-1α protein expression mostly via an enhanced receptor tyrosine kinase (RTK) signaling that induces the transcription of VEGF, VEGF receptor, platelet-derived growth factor (PDGF), and other angiogenic mediators (Zhong et al. 2000, Kim & Kaelin 2004, Fu et al. 2005). Androgens are shown to activate HIF-1α via autocrine RTK–PI3K/Akt signaling in PCa cells (Mabjeesh et al. 2003). In view of these facts, HIF-1α could be a logical chemopreventive target to control PCa tumor angiogenesis.

We have observed that silibinin inhibits hypoxia-induced expression of HIF-1α and VEGF in human PCa LNCaP and PC3 cells (unpublished data). A literature search shows that chemopreventive studies on HIF in PCa are limited. However, in other cancers, such as HepG2 human hepatoblastoma cells, torilin, a plant-derived sesquiterpene, downregulates hypoxia-induced expression of VEGF and IGF-II (Kim et al.
Flavopiridol is also shown to inhibit hypoxia-induced expression of VEGF in human neuroblastoma and monocytes (Melillo et al. 1999, Rapella et al. 2002). Nevertheless, more studies are needed to assess the effect of chemopreventive agents on HIF-1α, as well as HIF-1α-mediated expression of angiogenic factors in the suppression of PCa.

Cell adhesion molecules and matrix metalloproteinases (MMPs)

In the process of angiogenesis, endothelial or tumor cells show increased expression of αvβ3 and αvβ5 integrins, the heterodimeric cell-surface receptors, which is usually linked with the tumor grade (Fornaro et al. 2001, Slack-Davis & Parsons 2004). In radiotherapy, the activation of integrins causes radio-resistance in human PCa as reported in a PC3 xenograft experiment (Abdollahi et al. 2005). Inhibition of integrin expression prevents in vitro angiogenesis, and suppression of integrin function is shown to suppress tumor angiogenesis in mouse model (Abdollahi et al. 2005, Belvisi et al. 2005). The breakdown of tissue matrix to facilitate the movement of newly formed endothelial cells for vessel formation is required for angiogenesis. It is achieved by MMPs secreted by activated endothelial or tumor cells (Overall & Lopez-Otin 2002). MMP-2 and MMP-9 are overexpressed in invasive PCa and mediate the invasion of both tumor and endothelial cells (Overall & Lopez-Otin 2002, Mehta et al. 2003, Takaha et al. 2004). Together, these studies suggest that integrins and MMPs could be targeted by chemopreventive agents to check the invasive potential of PCa.

Aspirin is reported to inhibit adhesion of PC3 cells to fibronectin and vitronectin, which bind to integrin receptors (Lloyd et al. 2003). To our knowledge, there is no chemopreventive study on integrin signaling in PCa, and therefore, potential chemopreventive agents need to be investigated for their possible modulatory effect on this signaling pathway and its possible association with their efficacy against PCa. In other studies, both silymarin and silibinin have been shown to inhibit both secreted and cellular MMP-2 expression and suppress capillary formation by human umbilical vein endothelial cells (HUVEC) in Matrigel assay (Jiang et al. 2000, Singh et al. 2005a,b,c). EGCG also inhibits both MMP-2 and MMP-9 for its anti-angiogenic/metastatic activity in TRAMP model (Sartor et al. 2004). It also inhibits the PSA-induced degradation of basement membrane and activation of MMP-2 in cell culture (Pezzato et al. 2004). Recently, employing DU145 cells in culture and tumor xenograft models, curcumin has been shown to reduce the expression of MMP-2 and MMP-9 together with suppression in growth and invasive potential of tumor cells (Hong et al. 2006). Genistein also inhibits MMP-2 and MMP-9 and upregulates tissue inhibitor of metalloproteinases (TIMP)-1 leading to the suppression of vascular growth, tumor cell invasion, and bone metastasis (Li et al. 2004, Huang et al. 2005). RRR-α-tocopheryl succinate, a vitamin E derivative, inhibits MMPs activity and suppresses Matrigel invasion of PC3 and DU145 cells (Zhang et al. 2004). Luteolin and quercetin are also shown to inhibit MMP-2 and MMP-9 secretion from tumor cells (Huang et al. 1999). In addition to their effects on MMPs and TIMPs, the effect of chemopreventive agents should also be studied on endogenous angiogenesis inhibitors, such as angiostatin, endostatin, and platelet factor 4, which might also play an important role in an overall inhibitory effect of these agents on in vivo tumor angiogenesis. Furthermore, the discovery of specific vascular markers associated with tumor development could provide substantial success towards PCa chemoprevention via anti-angiogenic intervention.

Urokinase-type plasminogen activator (uPA)

In addition to neo-vascularization, tumor invasion involves enhanced degradation of extracellular matrix that facilitates tumor metastasis. Recent studies indicate that uPA, a serine protease, is causatively involved in the metastatic phenotype of many cancers, including PCa (Sheng 2001). Recently, valuable information has been generated regarding the role of uPA system in metastasis, where gene amplification has been identified as one of its mechanisms of overexpression in human PCa (Helenius et al. 2001). Animal PCa models evidently show the significance of uPA and uPA receptor (uPAR) in the invasion and metastases of tumors. The knockdown of uPA and uPAR expression by siRNAs in metastatic PCa cell-line PC3 has been shown to suppress tumor cell invasion (Pulukuri et al. 2005). In this study, the abrogation of uPA–uPAR signaling is reported to inhibit the activation of downstream molecular signaling targets, including ERK1/2 and Stat3. These reports suggest that chemopreventive intervention of uPA–uPAR signaling could be an alternative strategy to control PCa metastasis.

Green tea polyphenols are shown to inhibit uPA expression in TRAMP mice (Adhami et al. 2004). Genistein is reported to inhibit uPA production in PC3 cells (Skogseth et al. 2005). Retinoic acid inhibits the malignant potential, including extracellular proteolysis of PCa by inhibiting uPA expression (Webber &
Table 1 Potential molecular targets of the novel chemopreventive agent silibinin in prostate cancer (PCa)

Molecular alterations by silibinin in PCa

<table>
<thead>
<tr>
<th>Effect</th>
<th>Description</th>
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<tbody>
<tr>
<td>Suppression of androgen receptor (AR) nuclear translocation</td>
<td>Decrease in prostate specific antigen expression and secretion</td>
</tr>
<tr>
<td>Down-regulation of AR co-activator, the prostate epithelium-derived Ets transcription factor</td>
<td>Inhibition of ligand (transforming growth factor α, TGFα) binding to epithelial growth factor receptor (EGFR) and its internalization</td>
</tr>
<tr>
<td>Inhibition of Shc and extracellular signal-regulated kinase 1/2 activation</td>
<td>Decrease in urokinase-type plasminogen activator expression</td>
</tr>
<tr>
<td>Suppression of TGFα expression and secretion</td>
<td>Induction of insulin-like growth factor-binding protein-3 and its secretion</td>
</tr>
<tr>
<td>Inhibition of insulin receptor substrate-1 phosphorylation</td>
<td>Induction of Cip1/p21 and Kip1/p27 levels</td>
</tr>
<tr>
<td>Suppression of cyclin-dependent kinases and cyclins levels, and associated kinase activity</td>
<td>Increase in retinoblastoma protein (Rb/p110) hypophosphorylation</td>
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<tr>
<td>Increase in retinoblastoma protein (Rb/p110) hypophosphorylation</td>
<td>Decrease in serine and threonine phosphorylation of Rb/p110</td>
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<tr>
<td>Increase in hypophosphorylation of Rb/p107 and Rb2/p130</td>
<td>Decrease in E2F levels</td>
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<tr>
<td>Decrease in telomerase activity</td>
<td>Decrease in telomerase activity</td>
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<tr>
<td>Inhibition of inhibitory κBακBα kinase (IKKα) activity and an increase in κBα level</td>
<td>Inhibition of constitutive nuclear factor-κB (NF-κB) transciptional activity</td>
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<tr>
<td>Inhibition of tumor necrosis factor α-induced NF-κB transcriptional activity</td>
<td>Activation of ataxia telangiectasia-mutated kinase and checkpoint kinase 2</td>
</tr>
<tr>
<td>Activation of caspases</td>
<td>Increase in growth, survival, and migration of endothelial cells</td>
</tr>
<tr>
<td>Decrease in survivin expression (R P Singh &amp; R Agarwal unpublished observations)</td>
<td>Inhibition of urokinase-type plasminogen activator expression and activity</td>
</tr>
<tr>
<td>Decrease in vascular endothelial growth factor expression and secretion</td>
<td>Inhibition of cell growth and proliferation, and induction of differentiation morphology in PCa cells</td>
</tr>
<tr>
<td>Decrease in hypoxia-inducing factor-1α level (R P Singh &amp; R Agarwal unpublished observations)</td>
<td>Induction of strong G1 and G2–M cell-cycle arrest, and moderate apoptosis in PCa cells</td>
</tr>
<tr>
<td>Decrease in matrix metalloproteinase 2 activity in PCa and endothelial cells</td>
<td>Suppression of PCa tumor xenograft growth in athymic nude mice</td>
</tr>
<tr>
<td>Increase in growth, survival, and migration of endothelial cells</td>
<td>In vivo anti-proliferative and apoptotic effects in xenograft study</td>
</tr>
<tr>
<td>Inhibition of angiogenesis both in vitro and in vivo tumor xenograft</td>
<td>Inhibition of tumor growth in transgenic adenocarcinoma of the mouse prostate animal model (unpublished)</td>
</tr>
<tr>
<td>Inhibition of tumor growth in transgenic adenocarcinoma of the mouse prostate animal model (unpublished)</td>
<td>Non-toxic and physiologically available in plasma/tissues in animal and human studies</td>
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</table>

Inhibition of tumor growth in transgenic adenocarcinoma of the mouse prostate animal model (unpublished) Non-toxic and physiologically available in plasma/tissues in animal and human studies

Chemopreventive agents in PCa clinical trial

There are only few natural agents, which are under investigation for their chemopreventive efficacy in human PCa patients. However, epidemiological studies suggest that natural agents might have protective effects against PCa (Nelson et al. 2003). An epidemiological study with green tea consumption has been conducted in age-matched placebo and PCa patients with confirmed adenocarcinoma of the prostate in southeast China during 2001–2002 (Jian et al. 2004). In this study, tea consumption was limited to green tea and 55% of PCa patients were tea drinkers as compared with 80% in placebo. A decreased risk of PCa was noted with increasing frequency, duration, and quantity of green tea consumption. In a very recent randomized controlled trial, 200 mg thrice a day (total 600 mg/day) dose of a preparation of green tea catechins (GTCs: -epigallocatechin, 5.5%; -epicatechin, 12.24%; -epigallocatechin-3-gallate, 51.88%; -epicatechin-3-gallate, 6.12%; total GTCs, 75.7%; caffeine, < 1%) was given to men with high-grade PIN for 1 year (Bettuzzi et al. 2006). At the end of the study, there was only one tumor in the 30 GTCs-treated men, whereas nine tumors were observed in the 30 placebo-treated men. This study did not find any adverse effects of GTCs consumption. Another clinical trial of green tea (250 mg twice daily) in hormone refractory PCa patients (19 subjects) was found to have minimal clinical activity against hormone refractory PCa (Choan et al. 2005). However, this conclusion is based on the disease progression only in 15 patients, who completed at least 2 months of the treatment requirement. The α-tocopherol and β-carotene study, basically designed for the lung cancer chemoprevention, has shown that men, who received vitamin E for a 6-year period had a 34% lower incidence of PCa (Albanes et al. 1995). This finding has inspired an ongoing large multicenter trial, known as SELECT (selenium and vitamin E cancer prevention trial), with selenium and vitamin E for
PCa prevention; the results of this trial are still awaited (Klein et al. 2000). Silibinin is being investigated in phase I clinical study to assess its bioavailability and toxicity. The results obtained so far are promising to evaluate its efficacy and associated biomarkers in PCa patients in a pilot phase II clinical trial (Flagg et al. 2005). Overall, these epidemiological and clinical studies suggest the potential of natural agents in the chemoprevention of PCa; however, more studies are needed in the near future for their possible clinical applications.

Conclusions

To date, other than the fact that many targets are present on the membrane and inside the tumor cells, the outcome of an agent targeting a single molecule has been disappointing. The simplest reason could be that tumors have many molecular targets, which function aberrantly, and therefore, for the best results in the chemoprevention, as well as therapy of PCa, a multi-molecular targeting approach is essential. In this regard, as discussed above, chemopreventive agents, such as silibinin (Table 1), usually have many targets for their effective action in neoplastic cells, and therefore, these agents could be of potential use in controlling PCa malignancy. However, the modulatory effect of a chemopreventive agent on many cellular targets could most likely be a sequential or correlative effect, and therefore, knockout/in cell and animal models are now essential to further identify and establish critical molecular targets for the efficacy of chemopreventive agents, not in PCa but in other cancers as well. Such studies will advance the field of cancer chemopreventive drug development. So far, the recently identified chemopreventive agents, including silibinin, IP6, decursin, apigenin, and acacetin, along with green tea polyphenols, genistein, quercetin, and curcumin have shown immense potential for PCa control; however, more studies are needed for their possible clinical use against PCa.

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