Biochemical Pharmacology, Vol. 60, pp. 1051–1059, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00385-3

Cell Signaling and Regulators of Cell Cycle as Molecular Targets for Prostate Cancer Prevention by Dietary Agents

*Rajesh Agarwal**

CENTER FOR CANCER CAUSATION AND PREVENTION, AMC CANCER RESEARCH CENTER, DENVER, CO 80214, U.S.A.

ABSTRACT. Prostate cancer (PCA) is the most common invasive malignancy and leading cause (after lung) of cancer deaths in males. Since PCA is initially androgen-dependent, strategies are targeted toward androgen depletion for its control. However, tumor re-growth mostly occurs following this modality, and is androgen-independent. A loss of functional androgen receptor and an enhanced expression of growth factor receptors (e.g. erbB family members) and associated ligands have been shown to be the causal genetic events in PCA progression. These genetic alterations lead to an epigenetic mechanism where a feed-back autocrine loop between membrane receptor (e.g. epidermal growth factor receptor [erbB1] and associated ligand (e.g. transforming growth factor-α) results in an enhanced activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) as an essential component of the uncontrolled growth of PCA at an advanced and androgen-independent stage. Together, we rationalized that inhibiting these epigenetic events would be useful in controlling advanced PCA growth. Dietary polyphenolic flavonoids and isoflavones are being studied extensively as cancer-preventive and interventive agents. Therefore, we focused our attention on silymarin, genistein, and epigallocatechin 3-gallate (EGCG), present in milk thistle, soy beans, and green tea, respectively. The effect of these agents was assessed on the erbB1-Shc-ERK1/2 signal transduction pathway, cell cycle regulatory molecules, and cell growth and death. In androgen-independent human prostate carcinoma DU145 cells, silymarin, genistein, and EGCG resulted in a significant to complete inhibition of transforming growth factor-α-caused activation of membrane receptor erbB1 followed by inhibition of downstream cytoplasmic signaling target Shc activation and a decrease in its binding with erbB1, without an alteration in their protein expression. Silymarin and genistein also inhibited ERK1/2 activation, suggesting that these agents impair the activation of erbB1–Shc–ERK1/2 signaling in DU145 cells. In the case of EGCG, a further increase in ERK1/2 activation was observed that was related to its pro-oxidant and apoptotic activities. Silymarin, genistein, and EGCG also resulted in a significant induction of Cip1/p21 and Kip1/p27 and a decrease in cyclin-dependent kinase (CDK) 4, but a moderate inhibition of CDK2, cyclin D1, and cyclin E was observed. An enhanced level of Cip1/p21 and Kip1/27 also led to an increase in their binding to CDK4 and CDK2. Treatment of cells with silymarin, genistein, and EGCG also resulted in strong cell growth inhibition at lower doses, and complete inhibition at higher doses. In contrast to silymarin, higher doses of genistein also showed cell death. A more profound cytotoxic effect was observed in the case of EGCG, with strong cell death at lower doses and complete loss of viability at higher doses. Together, these results suggest that cell signaling and regulators of cell cycle are potential epigenetic molecular targets for prostate cancer prevention by dietary agents. More studies, therefore, are needed with these agents to explore their anticarcinogenic potential against human prostate cancer. BIOCHEM PHARMACOL **60**;8:1051–1059, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cell signaling; cell cycle regulators, silymarin, epigallocatechin 3-gallate; genistein; growth factor receptor; erbB1, MAPK, Cip1/p21; Kip1/p27

PROSTATE CANCER

Prostate cancer (PCA)† is the most common non-skin malignancy in United States males, accounting for 41% of newly diagnosed cases, and is the second leading cause (after lung) of cancer deaths [1]. The prostate is one of the accessory sex glands in males. PCA, however, is a proliferation of only prostatic epithelial cells and is predominantly located in the peripheral zone of the prostate; it can also occur in the transition zone. Despite the high incidence of PCA, little is known with certitude about its etiology. The induction of PCA in humans has been viewed as a multistage process (as outlined in Fig. 1) involving progression from low histologic grade small latent carcinoma to higher-grade large metastasizing carcinoma. It is becoming clear that in the genesis of PCA a variety of pathogenetic pathways exists. Among the widely accepted risk factors for PCA are age, race ethnicity, and geographical dependence

* Correspondence. Center for Cancer Causation and Prevention, AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214, U.S.A. Tel. +1-303-239-3580; FAX +1-303-239-3534; E-mail: agarwalr@amc.org

† *Abbreviations:* CDKs, cyclin-dependent kinases; CDKIs, cyclin-dependent kinase inhibitors; EGCG, epigallocatechin 3-gallate; EGFR (erbB1), epidermal growth factor receptor; ERK1/2, extracellular signal-regulated protein kinase 1/2; MAPK, mitogen-activated protein kinase; PCA, prostate cancer; RB, retinoblastoma; RTKs, receptor tyrosine kinases; and TGF-α, transforming growth factor-α.

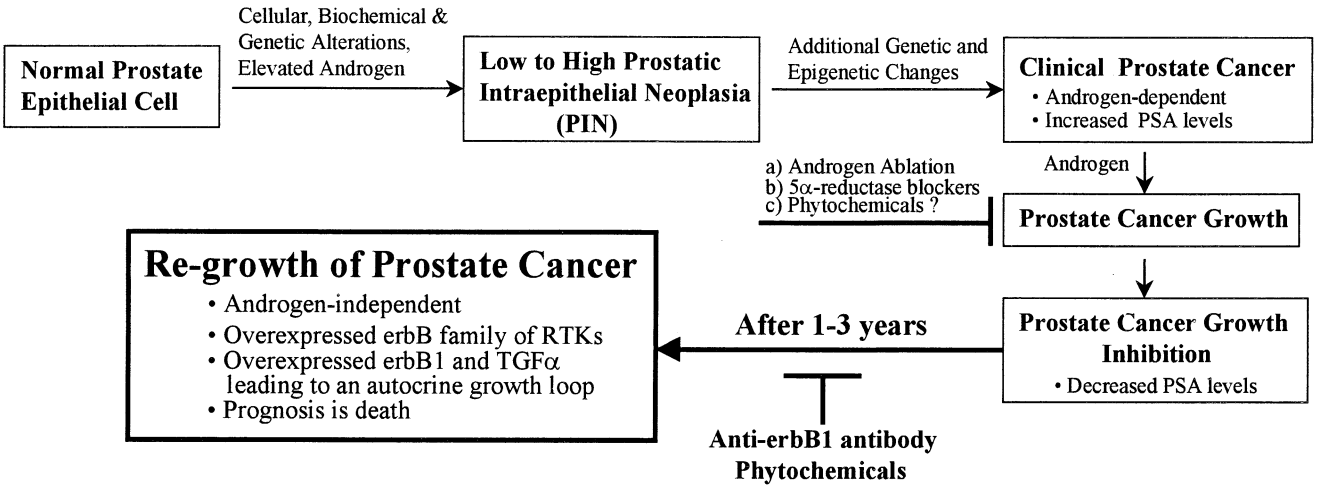

FIG. 1. Genesis of human prostate cancer is a multistep process. As shown in this figure, as an initial event, a normal epithelial prostate cell undergoes several cellular, biochemical, and genetic alterations leading to the formation of low- followed by high-grade prostatic intraepithelial neoplasia. The growth of transformed prostate epithelial cell is an elevated androgen-dependent phenomenon. Additional genetic and epigenetic changes in high-grade prostatic intraepithelial neoplasia lead to clinical prostate cancer that is androgen-dependent initially and can be monitored by an elevated serum prostate specific antigen (PSA) level. Since the malignancy is androgen-dependent at this stage, androgen deprivation and 5α-reductase blockers are extensively used to control and manage the disease at this point [5]. We have also reported that phytochemicals such as silymarin inhibit PSA levels regulated by both serum and androgen, causing strong inhibition of growth [43]. These approaches lead to the inhibition of PCA growth, which could be related to a decrease in serum PSA. Unfortunately, 1–3 years following these treatments, cancer re-growth mostly occurs that is totally androgen-independent and causally involves genetic alterations such as the overexpressed erbB family of RTKs and associated ligands. This situation leads to an epigenetic event of functional autocrine growth factor/receptor feed-back loop for the uncontrolled growth of malignant PCA cells as well as their metastasis to distant sites.

[2–4]. This malignancy is uncommon in the Asian population and high in the Scandinavian countries, with the highest incidence and mortality rates occurring in African American Males, the latter being twofold higher than in Caucasian American males [1–4]. Consistent with these reports, both epidemiology and laboratory studies have also suggested that diet and androgen alter PCA risk via a common etiologic pathway [2, 3]. It is becoming increasingly clear that androgens are involved in PCA pathogenesis and that cell division in the prostate is controlled by testosterone following intracellular conversion to its reduced form dihydrotestosterone [3] (Fig. 1).

The importance of testosterone in PCA could be further validated by studies showing that PCA rarely occurs in eunuchs or in men with a deficiency in 5α-reductase, the enzyme that converts testosterone to its active metabolite dihydrotestosterone [5]. Since cell division and proliferation in prostate is controlled by testosterone [5], inhibiting the biological effects of testosterone related to cellular proliferation in prostate tissue could be a novel approach for prevention against PCA. Indeed, androgen deprivation as well as 5α-reductase blockers have been extensively explored as a strategy for PCA prevention and therapy [5]. PCA patients treated with these therapies often experience remission of their PCA; however, tumor re-growth occurs which is largely due to progression of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation [6] (Fig. 1).

PROSTATE CANCER CHEMOPREVENTION

Since, even with the very high number of PCA diagnoses and related deaths in recent years, there are, other than surgery, no treatments for PCA in most patients, it is important that another strategy be developed. One approach is to prevent the occurrence of this disease in the first place. While many new classes of cancer chemopreventive agents are being evaluated in clinical trials for other malignancies, little success has been achieved in terms of PCA prevention. Chemoprevention of cancer is a means of cancer control where the occurrence of disease can be entirely prevented, slowed, or reversed by the administration of one or a combination of naturally occurring or synthetic compounds [7–12]. The overall goal of this modality is to reduce cancer incidence and multiplicity in the first place. The chemopreventive compounds are also known as anticarcinogens where the preventive approach includes intervention (or secondary prevention) of the conversion of precancerous lesions into malignant carcinomas [7]. Examples include prostatic intraepithelial neoplasia to prostate carcinomas, actinic keratosis to skin squamous cell carcinomas, etc. With regard to PCA prevention,

the preclinical efficacy of all-*trans*-*N*-(4-hydroxyphenyl-)retinamide, α-difluoromethylornithine, dehydroepiandrosterone, lirazole, lovastatin, oltipraz, finasteride (Proscar®), 9-*cis*-retinoic acid, etc., are being evaluated [13, 14]; all, however, are non-food-derived synthetic agents.

EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY OF RECEPTOR TYROSINE KINASES AND PROSTATE CANCER

The major goal of PCA research in recent years has been to elucidate and focus on the biology and molecular mechanisms of normal prostate and PCA to facilitate the design and conduct of molecular mechanism-based early phase prevention clinical trials. Indeed, several genetic alterations have been identified that lead to the induction and/or development of human PCA [13]. The early components of signal transduction pathways, specifically those of tyrosine kinases, were suggested to be of utmost significance for controlled cell growth and differentiation [15]. Ironically, a single genetic alteration in any of the cell signaling components results in continuous signaling, which in turn leads to uncontrolled cell growth (proliferation). RTKs participate in transmembrane signaling, whereas intracellular tyrosine kinases take part in signal transduction within the cell, including signaling to the nucleus [15]. Enhanced protein tyrosine kinase activity due to overexpression of RTKs and/or tyrosine kinase can lead to persistent stimulation by autocrinically secreted growth factors that in turn can lead to disease [15]. Enhanced activity of tyrosine kinases has been implicated in a wide variety of human malignancies; several studies have shown the increased expression of the EGFR or erbB family of RTKs in human malignancies, suggesting their role in the causation of such diseases [13, 15, 16]. With regard to human PCA, the aberrant expression of the erbB family of RTKs, such as EGFR (also known as erbB1), erbB2, and erbB3, has been demonstrated with strikingly high frequency in prostatic intraepithelial neoplasia and invasive PCA, both primary and metastatic [13, 17–19]. In addition, epidermal growth factor, TGF-α and erbB1 have been shown to be associated with the regulation of prostatic cell mitogenesis [20]. For example, hormone-independent prostate carcinoma cells commonly express high levels of erbB1 and TGF-α, thus making a functional autocrine feed-back loop for the hormone-independent growth of PCA [21, 22]. Employing the hormone-independent prostate carcinoma cell lines PC-3 and DU145, it has been shown that high-affinity, ligand-blocking monoclonal antibodies to erbB1 prevent its activation and also result in the growth inhibition of these cells [21–23]. Together, these studies imply that members of the erbB family of RTK-mediated signaling pathways may be contributory mechanisms for human PCA growth and metastasis [13, 16]; therefore, one practical and translational approach for the intervention of PCA could be to identify the inhibitors of the erbB family of RTK-mediated signaling pathway(s).

CELL CYCLE REGULATION

The significance of growth factors and the signaling pathway(s) initiated by them to regulate cell cycle progression in eukaryotes has been identified as an important component of their function [24–29]. Several studies have shown that cell signaling pathways determine cell growth as well as inhibition through cell cycle regulation [24–29]. However, cancer cells often display abnormalities in genes that govern the responses of these cells to external growth factors, growth factor receptors, proteins involved in the pathways of signal transduction in the cytoplasm, the nucleus, or both, and nuclear transcription factors [29]. In addition, defects in the regulation of cell cycle progression are thought to be one of the most common features of transformed cells [24]. Eukaryotic cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of CDKs at different phases [30–32]. The activities of CDKs are positively regulated by cyclins and negatively by CDKIs [25]. A cyclin–CDK complex hyperphosphorylates RB, leading to its release from E2F [24, 27–29]. The free transcription factor E2F then activates the genes responsible for cellular proliferation by progression through G1 phase [24, 27–29]. Impairment of a growth-stimulatory signaling pathway (such as erbB1, raf, MAPK) has been shown to induce the expression of CDKIs such as Cip1/p21 and Kip1/p27 [23, 26, 27, 33]. An induced CDKI binds to and subsequently inhibits cyclin–CDK activity, which interferes with hyperphosphorylation of RB by keeping it in the hypophosphorylated form and bound to E2F, thereby blocking cell proliferation and inducing cell growth arrest [24, 28, 29].

WORKING HYPOTHESIS

Taken together, the above-summarized studies clearly suggest that progression of PCA depends on both genetic and epigenetic events where the multistep process leads from transformation of normal prostate epithelial cell over an androgen-dependent non-metastatic phenotype to a highly malignant metastatic androgen-independent malignancy (Fig. 1). At this stage, a functional autocrine growth factor/receptor loop plays a causal role in disease progression, and the prognosis is death of the host (Fig. 1). Whereas it is equally important to focus on both genetic and epigenetic events associated with this malignancy, one could argue that other than gene therapy, corrections in genetic defects may perhaps be more difficult to achieve. Accordingly, the most practical and translational approach to control human PCA could be to explore epigenetic events as potential molecular targets for control, prevention, and intervention of this deadly malignancy. In this regard, it is important to highlight here that epidemiological studies have shown that even with the same incidence of latent small or non-infiltrating prostatic carcinomas, the incidence of clinical PCA and associated mortality is low in Japan and other Asian countries [34]. These epidemiolog-

ical data further support the hypothesis that although the initiation of PCA is inevitable, targeting the associated epigenetic events could control its progression to clinical cancer. For example, it can be argued that despite the same incidence of latent prostatic carcinomas, the incidence of clinical PCA is low in Asian countries because of their dietary habits, among which is a regimen rich in several flavonoids and isoflavones that inhibits the progression of clinical PCA by modulating epigenetic events [35, 36]. It is important to emphasize here that several dietary agents including flavonoids and isoflavones have been implicated in diet-related protection against cancer [7–12] and that dietary habits in Asian countries largely include consumption of yellow–green vegetables, fruits, soybeans, green tea, etc. [11, 35, 36].

Taken together, the elements in the above discussion lead to the working hypothesis that phytochemicals present in the diet humans consume routinely as well as those in dietary supplements could be strong preventive and interventive agents against PCA by modulating epigenetic events associated with the progression of latent prostatic neoplasia to clinical malignancy. Accordingly, we structured a working model (shown in Fig. 2) to assess the effect of phytochemicals on these events in advanced and androgen-independent human prostate carcinoma DU145 cells. In constructing this study model, our rationale was that inhibition of erbB1 activation (which is constitutively active in DU145 cells and in advanced PCA for that matter) will lead to inhibition of downstream signaling targets such as Shc (in a classical erbB1-Shc-Grb2/SOS-ras-raf-ERK1/2 signaling cascade) that will ultimately inhibit ERK1/2-mediated mitogenic signaling followed by an increase in CDKI, causing an inhibition of CDK activity. These effects will impair the hyperphosphorylation of RB, keeping it in the hyperphosphorylated form and bound to transcription factor E2F, thus leading to cell growth arrest. Initially, we focused our efforts on assessing the effect of silymarin using the model system detailed in Fig. 2. These studies (the results are detailed later) showed that treatment of prostate carcinoma DU145 cells with silymarin at 100–200-μM doses inhibits erbB1-Shc-ERK1/2 mitogenic signaling and modulates cell cycle regulators, leading to a G1 arrest and inhibition of cell growth and colony formation. Based on these exciting data, we next asked the question whether these important findings could be extended to other flavonoids and isoflavones with cancer-preventive potential such as EGCG and genistein. A brief description for the selection of these agents is provided in the following paragraph.

Fruits, vegetables, and common beverages, as well as several herbs and plants with diversified pharmacological properties, have been shown to be rich sources of microchemicals with the potential to prevent human cancers [7–12]. Among these, naturally occurring flavonoids and isoflavones have received increasing attention in recent years [7–12]. Accordingly, the major ongoing research project in our laboratory has been to assess the effect of the cancer-preventive phytochemicals silymarin (flavanone), genistein (isoflavone), and EGCG (flavanol) (Fig. 3) on epigenetic events involved in uncontrolled growth of advanced and androgen-independent PCA. Silymarin is a naturally occurring agent present in milk thistle (*Silybum marianum*) and is used clinically in Europe, and more recently in Asia and the United States, for the treatment of liver disease [37]. It is also sold as a dietary supplement in

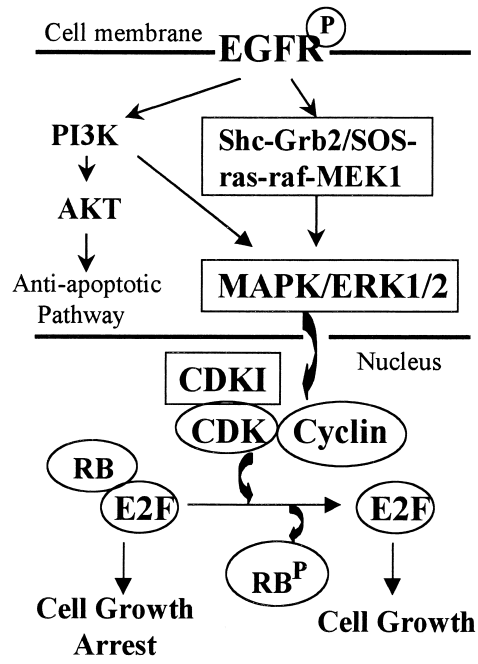

FIG. 2. A study model showing an interaction of mitogenic cell signaling with cell cycle regulators for cell growth. As shown in this figure, following EGFR activation (phosphorylation), several different mitogenic and cell survival pathways are activated in the cytoplasm. In a classical set-up, EGFR activation leads to recruitment of adoptor protein Shc followed by its activation and binding to Grb2/SOS that causes activation of ras-raf-MEK1-ERK1/2 mitogenic signaling [15, 62, 63]. Consistent with this signaling pathway and an autocrine growth factor/receptor feedback loop, MAPK/ERK1/2 is constitutively active in advanced and androgen-independent PCA, with this activation also causally associated with this malignancy at advanced stage [64, 65]. This and other mitogenic signaling cascades regulate cell cycle progression, possibly by activating CDKs that together with their catalytic subunit cyclins hyperphosphorylate RB, making transcription factor E2F free for cell growth [23–29, 33]. This model suggests that inhibition of erbB1 (EGFR) activation will lead to inhibition of the classical erbB1-Shc-Grb2/SOS-ras-raf-ERK1/2 signaling cascade that will ultimately inhibit ERK1/2-mediated mitogenic signaling, followed by an increase in CDKI and the resulting inhibition of CDK activity. These effects will impair the hyperphosphorylation of RB, keeping it in the hypophosphorylated form and bound to transcription factor E2F, thereby blocking cell proliferation and inducing cell growth arrest. Indeed, convincing evidence has been provided in recent studies from our laboratory and by other investigators that this is the case when phytochemicals and anti-EGFR antibody are used [23, 62, 66]. MEK1, MAPK kinase; P13K, phosphatidylinositol 3-kinase.

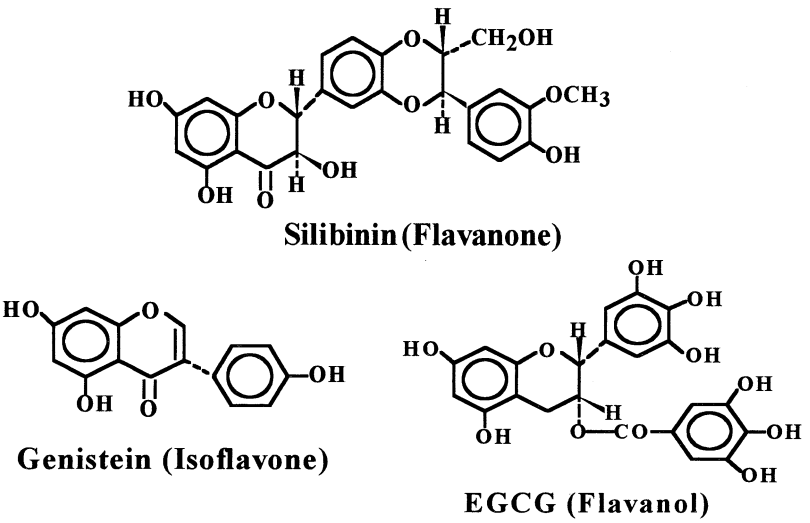

FIG. 3. Chemical structure of phytochemicals currently being studied for their efficacy on cell signaling and cell cycle regulators in human prostate carcinoma cells as summarized in the present overview.

the U.S.A. and Europe. Silymarin is non-toxic in acute, subchronic, and chronic tests in different animals and has no known LD_{50} [37]. In recent years, several studies from our laboratory have shown the cancer-preventive effects of silymarin in long-term tumorigenesis models and its anticarcinogenic activity in human prostate, breast, and cervical carcinoma cells [38–43]. Genistein is another dietary agent present in soybeans (*Glycine max*) [44] and has received much attention as a potential anticarcinogenic agent due to its effect on a number of cellular processes [45]. Several epidemiological and animal tumor studies have shown the preventive effects of genistein against various cancers [44, 46]. With regard to PCA, the anticarcinogenic and cancer-preventive effects of genistein have been extensively studied using cell and organ cultures and animal models; its efficacy is also being evaluated in PCA patients [47–55]. Tea (*Camellia sinensis*) is one of the most common beverages all over the world. Several studies from our group and by others have shown the cancer-preventive and anticarcinogenic effects of tea polyphenols on various cancers including skin, lung, esophagus, stomach, liver, intestine, pancreas, breast, and prostate [11, 12]. As a major component, EGCG constitutes ~50% (w/w) of the total green tea extract and has been implicated in both the cancer-preventive and anticarcinogenic effects of green tea [11, 12, 56–61].

EFFECT OF SILYMARIN ON EPIGENETIC EVENTS IN DU145 CELLS

For these studies, two different approaches were used. First, studies were done in serum-starved DU145 cells to assess the effect of ligand-caused activation of erbB1-Shc-MAPK/ERK1/2 mitogenic signaling, and secondly to determine the effect of silymarin on constitutive activation of the same signaling pathway and its association with cell cycle regulators. Whereas the first set of studies was useful in defining the mechanistic aspect of the study, the later studies represent the clinical PCA situation where these prostate carcinoma cells have autonomous growth advantage due to the ligand–receptor autocrine feed-back loop. In the first set of studies, treatment (with two medium changes at 12-hr intervals to remove ligand secreted via autocrine mechanism) of 36-hr serum-starved DU145 cells with silymarin resulted in a highly significant, dose-dependent inhibition of TGF-α-mediated activation of erbB1, but no change in its protein levels. Silymarin treatment of cells also resulted in a significant decrease in tyrosine phosphorylation of an immediate downstream target of erbB1, the adapter protein SHC, together with a decrease in its binding to erbB1. In other studies under similar treatment conditions, silymarin also showed a dose-dependent inhibition of TGF-α-caused activation of MAPK/ERK1/2 without any change in ERK1/2 protein levels. In additional studies, treatment of cells grown in 10% serum with different doses of silymarin also resulted in significant inhibition of constitutive tyrosine phosphorylation of both erbB1 and Shc followed by ERK1/2, but no change in their protein levels. Together, these results showed that silymarin impairs erbB1-Shc-ERK1/2-mediated mitogenic signaling in DU145 cells [62] (Fig. 4). In the erbB1-mediated mitogenic signaling pathway, via activation of Shc-Grb2-ras-raf, the ultimate cytoplasmic target is MAPK/ERK1/2, which following its activation translocates to the nucleus where it in turn activates transcription factors for cell growth and proliferation [15, 63]. In the case of advanced and androgen-independent PCA, several studies have shown genetic alterations resulting in an enhanced expression for erbB1 and associated ligand that leads to an epigenetic mechanism of autocrine growth loop via ligand/erbB1 interaction [20–23]. Together, these studies suggest that growth factors and receptors associated with PCA progression regulate cell growth mostly through the activation of MAPKs. Indeed, recent studies have shown that MAPK/ERK1/2 is constitutively very active in DU145 cells and that epidermal growth factor, insulin-like growth factor-1, and protein kinase A activator significantly activate

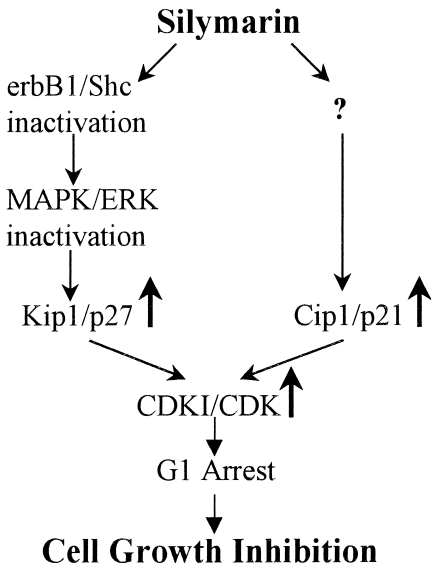

FIG. 4. A summary of the observed effects of silymarin on erbB-1-Shc-ERK1/2 signaling and cell cycle regulators in human prostate carcinoma DU145 cells. An inhibition of erbB1 activation by silymarin leads to a lack of Shc activation and binding to receptor followed by inhibition of ERK1/2 activation. This impairment of mitogenic signaling selectively induces Kip1/p27 followed by its increased binding to CDKs, causing an inhibition of their enzyme activity. This leads to an impairment of RB hyperphosphorylation, thereby keeping it hypophosphorylated and bound to E2F.* Together, these effects cause an arrest in cell cycle progression followed by cell growth inhibition.

MAPK/ERK1/2 in both LNCaP and DU145 human PCA cells via the erbB1 receptor [64]. In addition, an increase in the activation of MAPK/ERK1/2 signaling was also reported more recently as human PCA progresses to a more advanced and androgen-independent malignancy [65]. Consistent with the involvement of activated MAPK/ERK1/2, possibly via the TGF-α/erbB1 autocrine loop, in the progression of advanced and androgen-independent human PCA, the results of our study showing that impairment of erbB1-Shc activation results in the inhibition of MAPK/ERK1/2 activation in DU145 cells by silymarin could be of great significance in further evaluating the effect of this dietary supplement in human PCA prevention and/or intervention.

In the studies analyzing the effect of silymarin on cell cycle regulatory molecules, silymarin treatment of cells grown in 10% serum resulted in a significant induction of the CDKIs Cip1/p21 and Kip1/p27, concomitant with a significant decrease in CDK4 expression but no change in the levels of CDK2, CDK6, and associated cyclin E and cyclin D1. Cells treated with silymarin also showed an increased binding of CDKIs with CDKs, together with a marked decrease in the kinase activity of CDKs and associated cyclins. In additional studies, treatment of cells grown in 10% serum with anti-EGFR monoclonal antibody clone 225 also resulted in a significant inhibition of constitutive tyrosine phosphorylation of both erbB1 and Shc, but no change in their protein levels. Furthermore, whereas silymarin treatment resulted in a significant increase in the protein levels of both Cip1/p21 and Kip1/p27, monoclonal antibody 225 showed an increase only in Kip1/p27 [62]. These findings suggest that the observed effect of silymarin on an increase in CDKI protein levels is mediated via inhibition of erbB1 activation only in the case of Kip1/p27; however, additional pathways independent of inhibition of erbB1 activation are possibly responsible for the silymarin-caused increase in Cip1/p21 in DU145 cells [62] (Fig. 4). In other studies, silymarin treatment also induced a G1 arrest in the cell cycle progression of DU145 cells, and resulted in a highly significant to complete inhibition of both anchorage-dependent and -independent growth of DU145 cells in a dose- and time-dependent manner. As summarized in Fig. 4, together these results suggest that silymarin may exert a strong anticarcinogenic effect against PCA and that this effect is likely to involve impairment of the erbB1-Shc-ERK1/2-mediated mitogenic signaling pathway, leading to an induction of CDKIs that inhibit the growth-promoting activity of CDKs, causing G1 arrest followed by cell growth inhibition [62].

EFFECTS OF GENISTEIN AND EGCG ON EPIGENETIC EVENTS IN DU145 CELLS: A COMPARISON WITH SILYMARIN

Based on the above findings with silymarin, we next wondered whether these important findings could be extended to other flavonoids and isoflavones with cancer-preventive potential such as EGCG and genistein. For these studies, 36-hr serum-starved DU145 cells were treated with similar doses (100–200 μM) of silymarin, genistein, and EGCG for 2 hr followed by ligand TGF-α for 15 min, cell lysates were prepared, and levels of activated signaling molecules (erbB1-Shc-ERK1/2) were analyzed by immunoprecipitation and immunoblotting. As summarized in Table 1 [66], treatment of cells with silymarin, genistein, and EGCG at 100–200 μM resulted in a complete inhibition of TGF-α-caused activation of erbB1, followed by a moderate to strong inhibition of Shc activation as well as its binding to erbB1 without an alteration in their protein levels. Silymarin and genistein also inhibited ERK1/2 activation in a dose-dependent manner without an alteration in protein levels. These results suggested that these phytochemicals impair erbB1-Shc-ERK1/2 signaling in DU145 cells [66]. In the case of EGCG, it was interesting to observe that it inhibited erbB1-Shc activation but further stimulated ERK1/2 activation. At the same time as the detailed mechanism of this effect is currently under investigation in our laboratory, initial observations suggest that this effect of EGCG could be related to its pro-oxidant and apoptotic activities [67].

In other studies, we assessed the effect of these three

* Tyagi A, Agarwal C and Agarwal R, unpublished observation.

TABLE 1. Summary of the effects of the phytochemicals silymarin, genistein, and EGCG on cell signaling and cell cycle regulators, leading to biological responses in human prostate carcinoma DU145 cells [adopted from Refs. 62 and 66]

Biological Effect	Silymarin	Genistein	EGCG
ErbB1 activation	Inhibited	Inhibited	Inhibited
Shc activation	Inhibited	Inhibited	Inhibited
Shc binding to erbB1	Inhibited	Inhibited	Inhibited
MAPK/ERK1/2 activation	Inhibited	Inhibited	Induced
Cip1/p21 protein levels	Induced	Induced	Induced
Kip1/p27 protein levels	Induced	Induced	Induced
CDK2	Moderate inhibition	No effect	No effect
CDK4	Inhibited	Inhibited	Inhibited
CDK6	No effect	Not done	Not done
Cyclin D1	Weak inhibition	Weak inhibition	No effect
Cyclin E	Weak inhibition	Weak inhibition	No effect
CDKI/CDK binding	Induced	Induced	Induced
CDK and cyclin kinase activities	Inhibited	Not done	Not done
Cell cycle progression	G1 arrest	Not done	G1 arrest
Cell Growth	Inhibited	Inhibited	Inhibited
Apoptosis	Moderately induced	Induced	Induced
Soft agar colony formation	Inhibited	Not done	Not done
Nude mice xenograft growth	Inhibited	Not done	Not done

phytochemicals on cell cycle regulators and the growth and death of DU145 cells. In these studies, as summarized in Table 1 [66], silymarin, genistein, and EGCG resulted in a significant induction of Cip1/p21 and Kip1/p27 and a strong decrease in CDK4, but only a moderate effect on CDK2 and cyclins D1 and E. An enhanced level of CDKIs also led to an increase in their binding to CDK4 and CDK2. Treatment of cells with silymarin, genistein, and EGCG also resulted in 50–80% cell growth inhibition at lower doses and complete inhibition at higher doses. In contrast to silymarin, higher doses of genistein showed a cytotoxic effect causing 30–40% cell death. A more profound cytotoxic effect was observed in the case of EGCG, with 50% cell death at lower doses and a complete loss of viability at higher doses (Table 1).

CONCLUSION AND FUTURE GOALS

Concluding the overview provided in this communication, the convincing laboratory research findings summarized herein show an inhibitory effect of phytochemicals on erbB1-Shc-ERK1/2-mediated mitogenic signaling and on modulation of cell cycle regulators (specifically CDKIs, CDKs, cyclins, CDK activity) towards cell growth arrest and death. These studies form the basis for the question: what is the biological significance of the observed effect of these agents in terms of: a) human prostate carcinoma xenograft growth in nude mice; b) prostate cancer prevention and/or intervention in animals models; and c) human prostate cancer prevention/intervention in clinical trials? Currently, such studies are in progress in our program on "Phytochemicals and Cancer Prevention." However, based on studies already completed, it could be suggested that flavonoids and isoflavones are a class of dietary agents which should be studied in more detail to be developed as preventive and/or anticarcinogenic agents against human prostate cancer.

The studies summarized in this overview are supported by USPHS Grants CA83741 and CA64514, US Army Medical Research and Materiel Command Prostate Cancer Program DAMD17-98-1-8588, a grant from Ohio Cancer Research Associates through a subcontract with Case Western Reserve University, Cleveland, Ohio, and AMC Cancer Research Center Institutional Funds. The author also acknowledges that the work summarized here was done by Drs. Chapla Agarwal, Neehar Bhatia, Yogesh Sharma, and Xiaolin Zi.

References

1. Cancer facts and figures 1999. American Cancer Society, Inc., 2000.
2. Ross RK and Henderson BE, Do diet and androgens alter prostate cancer risk via a common etiologic pathway? *J Natl Cancer Inst* **86:** 252–254, 1994.
3. Wynder EL, Rose DP and Cohen LA, Nutrition and prostate cancer: A proposal for dietary intervention. *Nutrition and Cancer* **22:** 1–10, 1994.
4. Rose DP, Boyar AP and Wynder EL, International comparisons of mortality rates for cancer of the breast, ovary, prostate and colon and per capita food consumption. *Cancer* **58:** 2363–2371, 1986.
5. Aquilina JW, Lipsky JJ and Bostwick DG, Androgen deprivation as a strategy for prostate cancer chemoprevention. *J Natl Cancer Inst* **89:** 689–696, 1997.
6. Umekita Y, Hiipakka RA, Kokontis JM and Liao S, Human prostate tumor growth in athymic mice: Inhibition by androgens and stimulation by finasteride. *Proc Natl Acad Sci USA* **93:** 11802–11807, 1996.
7. Morse MA and Stoner GD, Cancer chemoprevention: Principles and prospects. *Carcinogenesis* **14:** 1737–1746, 1993.
8. Hong WK and Sporn MB, Recent advances in chemoprevention of cancer. *Science* **278:** 1073–1077, 1997.
9. Wattenberg LW, An overview of chemoprevention: Current status and future prospects. *Proc Soc Exp Biol Med* **216:** 133–141, 1997.

10. Ames BN, Gold LS and Willett WC, The causes and prevention of cancer. *Proc Natl Acad Sci USA* **92:** 5258–5265, 1995.
11. Yang CS and Wang ZY, Tea and cancer. *J Natl Cancer Inst* **85:** 1038–1049, 1993.
12. Agarwal R and Mukhtar H, Cancer chemoprevention by polyphenolic compounds present in green tea. *Drug News & Perspective* **8:** 216–225, 1995.
13. Karp JE, Chiarodo A, Brawley O and Kelloff GJ, Prostate cancer prevention: Investigational approaches and opportunities. *Cancer Res* **56:** 5547–5556, 1996.
14. McCormick DL, Rao KV, Steele VE, Lubet RA, Kelloff GJ and Bosland MC, Chemoprevention of rat prostate carcinogenesis by 9-*cis* retinoic acid. *Cancer Res* **59:** 521–524, 1999.
15. Levitzki A and Gazit A, Tyrosine kinase inhibition: An approach to drug development. *Science* **267:** 1782–1788, 1995.
16. Kelloff GJ, Fay JR, Steele VE, Lubet RA, Boone CW, Crowell JA and Sigman CC, Epidermal growth factor receptor tyrosine kinase inhibitors as potential cancer chemopreventives. *Cancer Epidemiol Biomarkers Prev* **5:** 657–666, 1996.
17. Bostwick DG. *c-erbB-2* oncogene expression in prostatic intraepithelial neoplasia: Mounting evidence for a precursor role. *J Natl Cancer Inst* **86:** 1108–1110, 1994.
18. Myers RB, Srivastava S, Oelschlager DK and Grizzle WE, Expression of p160erbB3 and p185erbB2 in prostatic intraepithelial neoplasia and prostatic adenocarcinoma. *J Natl Cancer Inst* **86:** 1140–1145, 1994.
19. Pretlow TG, Pelley RJ and Pretlow TP, Biochemistry of prostatic carcinoma. In: *Biochemical and Molecular Aspects of Selected Cancers* (Eds. Pretlow TG and Pretlow TP), pp. 169–237. Academic Press, San Diego, 1994.
20. Ware JL. Growth factors and their receptors as determinants in the proliferation and metastasis of human prostate cancer. *Cancer Metastasis Rev* **12:** 287–301, 1993.
21. Hofer DR, Sherwood ER, Bromberg WD, Mendelsohn J, Lee C and Kozlowski JM, Autonomous growth of androgen-independent human prostatic carcinoma cells: Role of transforming growth factor alpha. *Cancer Res* **51:** 2780–2785, 1991.
22. Fong CJ, Sherwood ER, Mendelsohn J, Lee C and Kozlowski JM, Epidermal growth factor receptor monoclonal antibody inhibits constitutive receptor phosphorylation, reduces autonomous growth, and sensitizes androgen-independent prostatic carcinoma cells to tumor necrosis factor alpha. *Cancer Res* **52:** 5887–5892, 1992.
23. Peng D, Fan Z, Lu Y, DeBlasio T, Scher H and Mendelsohn J, Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27KIP1 and induces G1 arrest in prostatic carcinoma cell line DU145. *Cancer Res* **56:** 3666–3669, 1996.
24. Hunter T and Pines J, Cyclins and cancer II: Cyclin D and CDK inhibitors come of age. *Cell* **79:** 573–582, 1994.
25. Morgan DO, Principles of CDK regulation. *Nature* **374:** 131–134, 1995.
26. Liu Y, Martindale JL, Gorospe M and Holbrook NJ, Regulation of p21WAF1/CIP1 expression through mitogen-activated protein kinase signaling pathway. *Cancer Res* **56:** 31–35, 1996.
27. Blagosklonny MV, Prabhu NS and El-Deiry WS, Defects in $p21^{WAF1/CIP1}$, Rb, and c-myc signaling in phorbol ester-resistant cancer cells. *Cancer Res* **57:** 320–325, 1997.
28. Grana X and Reddy P, Cell cycle control in mammalian cells: Role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CDKIs). *Oncogene* **11:** 211–219, 1995.
29. Weinstein IB, Relevance of cyclin D1 and other molecular markers to cancer chemoprevention. *J Cell Biochem Suppl* **25:** 23–28, 1996.
30. Draetta G, Cell cycle control in eukaryotes: Molecular mechanisms of cdc2 activation. *Trends Biochem Sci* **15:** 378–383, 1990.
31. Pines J and Hunter T, Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* **346:** 760–763, 1990.
32. Sherr CJ, G1 phase progression: Cycling on cue. *Cell* **79:** 551–555, 1994.
33. Wu X, Rubin M, Fan Z, DeBlasio T, Soos T, Koff A and Mendelsohn J, Involvement of p27KIP1 in G1 arrest mediated by an anti-epidermal growth factor receptor monoclonal antibody. *Oncogene* **12:** 1397–1403, 1996.
34. Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE and Mack TM, Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *Br J Cancer* **63:** 963–966, 1991.
35. Anderson KE, Rosner W, Khan MS, New MI, Pang S, Wissel PS and Kappas A, Diet–hormone interactions: Protein/carbohydrate ratio alters reciprocally the plasma levels of testosterone and cortisol and their respective binding globulins in man. *Life Sci* **40:** 1761–1768, 1987.
36. Greco KE and Kulawiak L, Prostate cancer prevention: Risk reduction through life-style, diet, and chemoprevention. *Oncol Nurs Forum* **21:** 1504–1511, 1994.
37. Agarwal R, Katiyar SK, Lundgren DW and Mukhtar H, Inhibitory effect of silymarin, an anti-hepatotoxic flavonoid, on 12-*O*-tetradecanoylphorbol-13-acetate-induced epidermal ornithine decarboxylase activity and mRNA in SENCAR mice. *Carcinogenesis* **15:** 1099–1103, 1994.
38. Katiyar SK, Korman NJ, Mukhtar H and Agarwal R, Protective effects of silymarin against photocarcinogenesis in mouse skin model. *J Natl Cancer Inst* **89:** 556–566, 1997.
39. Zi X, Mukhtar H and Agarwal R, Novel cancer chemopreventive effects of a flavonoid antioxidant silymarin: Inhibition of mRNA expression of an endogenous tumor promoter TNFα. *Biochem Biophys Res Commun* **239:** 334–339, 1997.
40. Lahiri-Chatterjee M, Katiyar SK, Mohan RR and Agarwal R, A flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in SENCAR mouse skin tumorigenesis model. *Cancer Res* **59:** 622–632, 1999.
41. Zi X, Feyes DK and Agarwal R, Anti-carcinogenic effect of a flavonoid antioxidant silymarin in human breast cancer cells MDA-MB 468: Induction of G1 arrest through an increase in Cip1/p21 concomitant with a decrease in kinase activity of CDKs and associated cyclins. *Clin Cancer Res* **4:** 1055–1064, 1998.
42. Ahmad N, Gali H, Javed S and Agarwal R, Skin cancer chemopreventive effects of a flavonoid antioxidant silymarin are mediated via impairment of receptor tyrosine kinase signaling and perturbation in cell cycle progression. *Biochem Biophys Res Commun* **247:** 294–301, 1998.
43. Zi X and Agarwal R, Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention. *Proc Natl Acad Sci USA* **96:** 7490–7495, 1999.
44. Messina MJ, Persky V, Setchell KD and Barnes S, Soy intake and cancer risk: A review of the *in vitro* and *in vivo* data. *Nutr Cancer* **21:** 113–131, 1994.
45. Shao ZM, Wu J, Shen ZZ and Barsky SH, Genistein exerts multiple suppressive effects on human breast carcinoma cells. *Cancer Res* **58:** 4851–4857, 1998.
46. Barnes S, Peterson TG and Coward L, Rationale for the use of genistein-containing soy matrices in chemoprevention trials for breast and prostate cancer. *J Cell Biochem Suppl* **22:** 181–187, 1995.

47. Peterson G and Barnes S, Genistein and biochanin A inhibit the growth of human cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* **22:** 335–345, 1993.
48. Onozawa M, Fukuda K, Ohtani M, Akaza H, Sugimura T and Wakabayashi K, Effects of soybean isoflavones on cell growth and apoptosis of the human prostatic cancer cell line LNCaP. *Jpn J Clin Oncol* **28:** 360–363, 1998.
49. Davis JN, Singh B, Bhuiyan M and Sarkar FH. Genistein-induced upregulation of p21WAF1, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. *Nutr Cancer* **32:** 123–131, 1998.
50. Dalu A, Haskell JF, Coward L and Lamartiniere CA, Genistein, a component of soy, inhibits the expression of the EGF and ErbB2/Neu receptors in the rat dorsolateral prostate. *Prostate* **37:** 36–43, 1998.
51. Geller J, Sionit L, Partido C, Li L, Tan X, Youngkin T, Nachtsheim D and Hoffman RM, Genistein inhibits the growth of human patient BPH and prostate cancer in histoculture. *Prostate* **34:** 75–79, 1998.
52. Landstrom M, Zhang JX, Hallmans G, Aman P, Bergh A, Damber JE, Mazur W, Wahala K and Adlercreutz H, Inhibitory effects of soy and rye diets on the development of Dunning R3327 prostate adenocarcinoma in rats. *Prostate* **36:** 151–161, 1998.
53. Zhou JR, Gugger ET, Tanaka T, Guo Y, Blackburn GL and Clinton SK, Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. *J Nutr* **129:** 1628–1635, 1999.
54. Strom SS, Yamamura Y, Duphorne CM, Spitz MR, Babaian RJ, Pillow PC and Hursting SD, Phytoestrogen intake and prostate cancer: A case-control study using a new database. *Nutr Cancer* **33:** 20–25, 1999.
55. Moyad MA, Soy, disease prevention, and prostate cancer. *Semin Urol Oncol* **17:** 97–102, 1999.
56. Dong Z, Ma W, Huang C and Yang CS, Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (−)-epigallocatechin-3 gallate, and theaflavins. *Cancer Res* **57:** 4414–4419, 1997.
57. Ahmad N, Feyes DK, Nieminen A, Agarwal R and Mukhtar H, Green tea constituent epigallocatechin-3 gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst* **89:** 1881–1886, 1997.
58. Yang Y, Liao J, Kim K, Yurkow EJ and Yang CS, Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* **19:** 611–616, 1998.
59. Paschka AG, Butler R and Young CY, Induction of apoptosis in prostate cancer cell lines by the green tea component, (−)-epigallocatechin-3 gallate. *Cancer Lett* **130:** 1–7, 1998.
60. Liang Y, Chen Y, Lin Y, Lin-Shiau S, Ho C and Lin J, Suppression of extracellular signals and cell proliferation by the black tea polyphenol, theaflavin-3,3′-digallate. *Carcinogenesis* **20:** 733–736, 1999.
61. Ahn H, Hadizadeh KR, Seul C, Yun Y, Vetter H and Sachinidis A, Epigallocatechin-3 gallate selectively inhibits the PDGF-BB-induced intracellular signaling transduction pathway in vascular smooth muscle cells and inhibits transformation of sis-transfected NIH 3T3 fibroblasts and human glioblastoma cells (A172). *Mol Biol Cell* **10:** 1093–1104, 1999.
62. Zi X, Grasso AW, Kung HJ and Agarwal R, A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G1 arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. *Cancer Res* **58:** 1920–1929, 1998.
63. Grasso AW, Wen D, Miller CM, Rhim JS, Pretlow TG and Kung H-J, ErbB kinases and NDF signaling in human prostate cancer cells. *Oncogene* **15:** 2705–2716, 1997.
64. Putz T, Culig Z, Eder IE, Nessler-Menardi C, Bartsch G, Grunicke H, Uberall F and Klocker H, Epidermal growth factor (EGF) receptor blockade inhibits the action of EGF, insulin-like growth factor I, and a protein kinase A activator on the mitogen-activated protein kinase pathway in prostate cancer cell lines. *Cancer Res* **59:** 227–233, 1999.
65. Gioeli D, Mandell JW, Petroni GR, Frierson Jr HF and Weber MJ. Activation of mitogen-activated protein kinase associated with prostate cancer progression. *Cancer Res* **59:** 279–284, 1999.
66. Bhatia N, Zi X and Agarwal R, Enhanced polyphenolic nature of flavonoid antioxidants plays a detrimental role in the activation of survival factor Akt and apoptosis in prostate cancer DU145 cells: A comparison of silymarin, genistein and EGCG. *Proc Am Assoc Cancer Res* **40:** 533, 1999.
67. Bhatia N and Agarwal R, Hydrogen peroxide-mediated prooxidant activity of epigallocatechin 3-gallate in human prostate carcinoma DU145 cells: Effect on cell growth and viability, and MAP kinases. *Proc Am Assoc Cancer Res* **41:** 533, 2000.