

The Control of Prostate-Specific Antigen Expression and Gene Regulation by Pharmacological Agents

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Abstract	73
I. Introduction	74
II. Model systems for studying prostate-specific antigen regulation	74
III. Overview of prostate-specific antigen gene regulation by the androgen receptor	75
IV. Agents that up-regulate prostate-specific antigen	75
A. Thalidomide	75
B. TNP-470	75
C. Granulocyte macrophage colony-stimulating factor	76
D. Phenylacetate	76
E. Butyrate and its analogs	77
1. Butyrate	77
2. Phenylbutyrate	77
3. Isobutyramide	77
F. Vitamin D ₃ and synthetic vitamin D analogs	78
V. Agents that down-regulate prostate-specific antigen	78
A. Gallium nitrate	78
B. Troglitazone	79
C. Carboxyamido-triazole	79
D. Finasteride	79
E. Leuprolide acetate	80
F. PC-SPES	80
G. Suramin	81
H. Flavopiridol	82
I. Estramustine phosphate and its metabolites	82
J. Resveratrol	82
VI. Agents that have a dual effect on prostate-specific antigen	83
A. Retinol, 9- <i>cis</i> -retinoic acid, and 13- <i>cis</i> -retinoic acid	83
B. All- <i>trans</i> -retinoic acid	83
C. <i>N</i> -(4-hydroxyphenyl)retinamide	85
VII. Discussion	86
Acknowledgments	87
References	87

Abstract—Prostate-specific antigen is a serine protease that is a member of the kallikrein family. It is widely used as an indicator of tumor burden and as a surrogate marker for disease progression in men with androgen-independent prostate cancer. It has been shown that the expression and/or secretion of this glycoprotein can be regulated by pharmacological agents. The effects of these agents on PSA may be independent of

their effects on cell growth. For example, a pharmacological agent may down-regulate PSA expression/secretion but have no effect on tumor cell growth. In this case, a patient receiving this therapeutic agent might be falsely considered as having a clinical response. Alternatively, an agent might up-regulate PSA expression/secretion and have an inhibitory effect on cell growth. A patient receiving this therapeutic agent might be diagnosed with progressive disease unless an alternative method for assessing tumor burden is used. Thus, when an agent is to be evaluated in a clinical trial utilizing PSA as a marker for disease

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progression, it is important to prospectively test whether the agent has an effect on PSA expression and/or secretion. In addition, it is equally important to understand how these regulatory effects relate to cell growth. The purpose of this review is to describe sev-

eral agents that have been tested for their regulatory effects on PSA and to discuss potential mechanisms of by which this regulation may occur. The implications of these findings in the evaluation of new agents in androgen-independent prostate cancer will be considered.

I. Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer death of men in the United States. In 1999, it is estimated that 179,300 new cases were diagnosed and 37,000 deaths occurred from this disease (Landis et al., 1999). The cornerstone for treatment of metastatic prostate cancer is androgen ablation, which is typically achieved through either medical or surgical castration (Huggins and Hodges, 1941). This therapeutic maneuver leads to a favorable response and disease regression in greater than 80% of the patients. However, within 12 to 18 months, the majority of men with metastatic disease will develop androgen-independent growth, progressive disease, and will ultimately succumb to their disease (The Veterans Administration Co-operative Urological Research Group, 1967; Crawford et al., 1989; The Canadian Anandron Study Group, 1990; Denis et al., 1993; Janknegt et al., 1993; Bertagna et al., 1994). The treatment of androgen-independent prostate cancer (AIPC²) is problematic and, for the most part, has been predominantly palliative. Conventional cytotoxic regimens have provided little or no benefit in prostate cancer, yielding response rates between 10 and 20% (Eisenberger et al., 1985, 1987a,b; Eisenberger and Abrams, 1988; Panvichian and Pienta, 1996; Colleoni et al., 1997). Thus, the pursuit of new treatment options and pharmacological agents that are effective against AIPC is an area of active research.

Prostate-specific antigen (PSA) is a 33-kDa glycoprotein and a member of the kallikrein family of serine proteases (Clements, 1989). It is secreted by normal, hyperplastic, and cancerous prostatic epithelia. One of its roles is to degrade high molecular weight seminal vesicle proteins that otherwise would form seminal coagulates (Allhoff et al., 1983; Lilja, 1985; Leo et al., 1991). Alternatively, it appears to be involved in prostate growth regulation by cleaving insulin-like growth

factor-binding proteins and thereby increasing the bioavailability of insulin-like growth factors (Doherty et al., 1999; Sutkowski et al., 1999). Elevated levels of PSA occur in patient sera in cases of prostate cancer, benign prostatic hyperplasia, and prostatitis (Gittes, 1991). PSA is a sensitive indicator of tumor burden (Chybowski et al., 1991). It is regarded as a reliable surrogate marker for survival and disease progression for patients with AIPC (Ferro et al., 1989; Kelly et al., 1993; Thibault et al., 1993; Fossa and Pause, 1994; Sridhara et al., 1995; Bubley et al., 1999). In 1989, clinical trials began utilizing PSA as an indicator of tumor burden and most trials continue to monitor PSA (Ferro et al., 1989). Figg et al. (1996) found that approximately 90% of patients with advanced metastatic prostate cancer have elevated PSA. This same group reported a median survival of 19.0 months versus 6.3 months for patients that experienced a 50% PSA decline versus those that did not (Thibault et al., 1993). Kelly and colleagues (1983) found a median survival of greater than 25 months in those patients that exhibited a greater than 50% decrease in PSA following an investigational regimen versus 8.6 months in those patients that did not achieve that level. From these data, PSA has been validated as an important diagnostic marker for prostate carcinoma and as a highly useful surrogate marker for patients with prostate cancer.

The growing body of literature has raised the concern that some investigational agents may affect PSA expression or secretion independently from alterations in tumor growth or volume. The purpose of this review is to describe several agents that have been tested for their ability to regulate PSA and to discuss potential mechanisms by which this regulation may occur. The effects of androgens and antiandrogens on the regulation of PSA are discussed elsewhere and will not be addressed (Gleave et al., 1986; Goldfarb et al., 1986; Young et al., 1991; Henttu et al., 1992; Lee et al., 1994; Luke and Coffey, 1994; Dai et al., 1996). The implications of these findings in the evaluation of new agents in AIPC will be considered.

II. Model Systems for Studying Prostate-Specific Antigen Regulation

There are a limited number of cell lines and model systems available for the study of this disease. One of

² Abbreviations: AIPC, androgen-independent prostate cancer; PSA, prostate-specific antigen; AR, androgen receptor; SRBC, steroid receptor binding consensus sequence; GM-CSF, granulocyte macrophage colony-stimulating factor; TNF, tumor necrosis factor; IL-1, interleukin-1; PA, phenylacetate; PB, phenylbutyrate; DHT, dihydrotestosterone; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; FBS, fetal bovine serum; CSS, charcoal-stripped serum; PPAR γ , peroxisome proliferator-activated receptor γ ; CAI, carboxyamido-triazole; LHRHa, luteinizing hormone-releasing hormone analog; EGF, epidermal growth factor; PCNA, proliferating cell nuclear antigen; 4-HPR, *N*-(4-hydroxyphenyl)retinamide; RA, retinoic acid; RAR α , retinoic acid receptor α ; RXR α , retinoic X receptor α ; hKLK2, human kallikrein 2.

the most commonly used models is the human prostatic epithelial cell line LNCaP. It was originally derived from a lymph node metastasis of prostate carcinoma and is androgen-sensitive and secretes PSA (Horoszewicz et al., 1983). In addition to expressing PSA, LNCaP cells also express a functional, albeit mutant, androgen receptor (AR). The LNCaP AR has a point mutation in the steroid binding domain at codon 877. As a consequence, progesterone and estrogen as well as anti-androgens such as hydroxyflutamide can activate this receptor (Veldscholte et al., 1990a,b; Klocker et al., 1994; Figg et al., 1995). Two other human prostate cell lines that are widely used are PC-3 and DU145, derived from a bone and a brain metastasis of prostate carcinoma, respectively (Stone et al., 1978; Kaighn et al., 1979). Both cell lines are androgen-independent, do not secrete PSA, nor express AR.

Although cell lines are good model systems for the in vitro evaluation of pharmacological agents, a major disadvantage is that they may not accurately reflect the clinical situation. For this reason, many researchers have relied on animal xenografts to provide a more realistic view of the activity of therapeutic agents. All of the above-mentioned tumor cell lines are tumorigenic in athymic nude mice. In addition, the LuCaP 23 tumor lines are often used in in vivo evaluation of potential therapeutic agents. These tumor lines were established from the metastases of a patient with AIPC. They secrete PSA, are androgen sensitive, and produce a functional AR (Ellis et al., 1996). Several other model systems are available for the study of prostate cancer and have been extensively reviewed by Navone et al. (1999). However, most are not suitable for the study of PSA expression and regulation.

III. Overview of Prostate-Specific Antigen Gene Regulation by the Androgen Receptor

The AR is responsible for the transactivation of PSA by binding to a steroid receptor-binding consensus sequence (SRBC) in the promoter region of this gene. Binding of the AR to the SRBC leads to up-regulation of the transcriptional activity of the PSA gene (Luke and Coffey, 1994; Cleutjens et al., 1996). It has been shown that expression of AR parallels the expression of PSA mRNA (Young et al., 1991; Goldfarb et al., 1986; Gleave et al., 1992), PSA glycoprotein during development (Goldfarb et al., 1986), as well as the growth of LNCaP tumors in nude mice (Gleave et al., 1992). Although the AR appears to be the major influence on the transcriptional transactivation of PSA, PSA gene expression has also been shown to be regulated by various growth factors and the extracellular matrix (Guo et al., 1994; Sica et al., 1999).

IV. Agents That Up-Regulate Prostate-Specific Antigen

A. Thalidomide

Thalidomide (*N*-phthalidoglutarimide) was originally marketed as a sedative and as an antiemetic in the 1950s. Although thalidomide showed no toxicity in rodents, it was discovered to be a potent teratogen in humans and was withdrawn from the market (McBride, 1961; Lenz, 1962). However, thalidomide remains a useful pharmacological agent and has proven therapeutic value for a variety of human pathologies (Kluken and Wentz, 1974; Vincente et al., 1993; Sharpstone et al., 1995). It is currently being tested as an antiangiogenic agent, and a phase II clinical trial for patients with AIPC has been completed recently (D'Amato et al., 1994; W. D. Figg, W. Dahut, P. Duray, M. Hamilton, A. Tompkins, S. Steinberg, E. Jones, A. Premkumar, M. Linehan, M. K. Floeter, et al., submitted for publication). Dixon et al. (1999) exposed the LNCaP and PC-3 cell lines to clinically achievable concentrations of thalidomide. The number of viable cells and the amount of PSA secreted into the supernatant (LNCaP only) were measured daily. After 120 h of treatment, both cell lines showed an approximate 20% decrease in cell number compared with controls. On the contrary, the amount of PSA secreted by LNCaP was increased at a statistically significant level. Thus, thalidomide can alter PSA secretion. The effect of thalidomide on the transcription or translation of PSA was not tested; however, preliminary data from cDNA expression arrays suggested that it could modulate the expression of several genes at a transcriptional or post-transcriptional level. Whether thalidomide modulates PSA gene expression could not be ascertained from the cDNA expression arrays used since this cDNA was not present on the arrays.

Thalidomide is a very unstable compound that hydrolyzes readily at neutral pH (Huupponen and Pyykko, 1995; Simmons et al., 1997). In the experiments described above, thalidomide underwent no metabolic breakdown as would occur in patients. Bauer et al. (1998) have shown that metabolic activation of thalidomide is species-dependent and that the metabolites generated in rodent systems are different from those generated in humans. Unfortunately, most of the currently available prostate cancer models are in rodents, making it impossible to test the activity of thalidomide in human tumor xenografts.

B. TNP-470

TNP-470 and its metabolite, AGM-1883, are synthetic agents that were identified as more potent and less toxic inhibitors of angiogenesis and tumor growth than fumagillin (Ingber et al., 1990). Treatment of LNCaP cells in vitro for 5 days showed a moderate reduction in cell proliferation by both TNP-470 and AGM-1883 (Horti et al., 1999). Accompanying inhibition of cell proliferation,

TNP-470 caused a 10 to 50% increase in the amount of PSA secreted per cell. AGM-1883 also showed an increase in PSA secretion per cell of 30 to 70%. No concentration dependence was observed with AGM-1883. It has been proposed that inhibition of endothelial cell growth and the antiangiogenic properties of TNP-470 stem from its ability to inhibit growth factor-induced DNA synthesis. TNP-470 has also been shown to regulate the transcription of specific *cdk* and cyclin gene families (Kusaka et al., 1991; Kato et al., 1994). These aspects of TNP-470 may be a potential mechanism of action for its growth inhibitory effects in tumor cells. The increase in PSA secretion by TNP-470 was reflected by equivalent increases of intracellular PSA protein and PSA mRNA. TNP-470 transiently up-regulated AR transcription, similar to PSA, suggesting that increased AR levels could account for the increased expression of PSA. Thus, control of PSA secretion by TNP-470 appears to be at a transcriptional and possibly at a pretranslational level. The control of PSA by TNP-470 appears to be regulated through the AR.

In the phase I trial of TNP-470 in patients with AIPC, Logothetis et al. (2001) showed reversible increases in serum PSA levels upon discontinuation of therapy. Sartor (1995) showed a similar withdrawal phenomenon. The observations of these two reports suggest that the *in vitro* effects noted by Horti et al. (1999) are reflected clinically, and in the case of TNP-470, reliance on PSA as a surrogate marker of tumor progression is compromised.

C. Granulocyte Macrophage Colony-Stimulating Factor

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a cytokine whose antitumor activity may be mediated through the induction of systemic immune responses. These immune responses are instigated through the indirect activation of T cells via the induction of tumor necrosis factor (TNF) and interleukin-1 (IL-1) as well as through the activation of the antitumor activity of macrophage and dendritic cells (Fagerberg, 1996; Thomas and Lipsky, 1996). The potential of GM-CSF as a therapeutic agent in prostate cancer was studied using the rat Dunning model of prostate cancer (Viewig et al., 1994). It was demonstrated that rats vaccinated with irradiated prostate cancer cells that secreted GM-CSF had a longer period of disease-free survival than animals that received mock-transfected cells and injections of GM-CSF. When Small et al. (1999) evaluated the activity of GM-CSF against LNCaP cells, it was found to have a cytostatic effect on cell growth while producing an 11.2 to 72.3% increase in secreted PSA. Further evaluation of GM-CSF showed that, although PSA secretion was increased, there was actually a modest reduction in the amount of both intracellular PSA and PSA transcripts. The reduction in PSA transcription was accompanied by a similar reduction in the amount of AR suggesting that GM-CSF regulates PSA

expression at a transcriptional level through its effects on AR and at a post-translational level.

The initial results of a phase II study of GM-CSF in men with AIPC showed that upon administration of GM-CSF, serum PSA values declined followed by elevation during the off-therapy period. These results are the opposite of those observed with TNP-470 by Logothetis et al. (2000). In this study, the PSA levels dropped during the off-therapy period and rose while the patients received drug. Although 5 of 22 patients showed a greater than 50% decrease in PSA on at least one occasion, these declines were not sustained. The trial was modified and PSA oscillations were less obvious. Ten of 11 men evaluated demonstrated a median decrease in PSA of 37%; one patient experienced a sustained PSA decline of greater than 50% for more than 6 weeks that was accompanied by an improvement on bone scan. From these results, it appears that the effects of GM-CSF on PSA regulation are complex and occur at multiple levels. The use of PSA as a marker for disease progression with this agent is questionable.

D. Phenylacetate

Phenylacetate (PA) is an aromatic fatty acid that is a metabolite of the phenylalanine pathway. It has been shown to have differentiating properties in many cancer cell lines including prostate, breast, melanoma, medulloblastoma, and astrocytoma (Samid et al., 1992, 1993; Liu et al., 1994; Stockhammer et al., 1995; Adam et al., 1996a,b; Esquenet et al., 1996). Samid et al. (1993) demonstrated that PA has a selective cytostatic effect for prostate carcinoma cell lines, but not for normal endothelial cells or skin fibroblasts. The combination of inducing differentiation and inhibiting tumor cell proliferation made PA an interesting agent for investigation. Most tumor cells are thought to be less differentiated than normal cells, and it was hoped that promotion of differentiation would halt or reverse the malignant process.

Several groups have reported that the well differentiated prostate cancers actually secrete more PSA per cell than those that are less differentiated (Stein et al., 1982; Ellis et al., 1984; Svanholm, 1986). These observations prompted an investigation regarding the *in vitro* and *in vivo* effects of PA on prostate cell growth and PSA production (Walls et al., 1996). These investigators found that although treatment of LNCaP cells with 3 mM to 10 mM PA resulted in an inhibition of cell proliferation, there was a 3- to 4-fold increase in the amount of PSA secreted per cell. Immunohistochemical analysis of LNCaP xenografts grown in male nude mice also showed a 4-fold increase in the number of PSA-producing cells and a reduction in mitotic figures in treated versus control animals. However, it is not stated whether the PA-treated tumors were more differentiated than the untreated controls. RNA blot analysis showed an increase of PSA transcripts upon treatment with 5 mM PA for 3

days, indicating that up-regulation of PSA secretion appears to begin at a transcriptional or pretranslational level. The results of the RNA blot were not quantitated, so it is unclear whether there are additional levels of control that may be modulated by PA. Since PA was shown to increase PSA secretion while producing a cytostatic effect on cell growth both in an in vitro and an in vivo model system, interpretation of a patient's response to PA was problematic based on the use of serum PSA levels alone.

E. Butyrate and Its Analogs

1. *Butyrate.* Butyrate is a naturally occurring, short-chain fatty acid that is a potent inducer of cellular differentiation. The effects on differentiation are mediated through its inhibition on histone deacetylase (Candido et al., 1978). Inhibition of this enzyme leads to an increase in histone acetylation, changes in chromosome structure, and increased DNA transcription (Candido et al., 1978; Norton et al., 1989). Several investigators have tested the effects of sodium butyrate on PSA expression. The consensus appears to be that butyrate causes a significant increase, 3- to 4-fold, in PSA secretion in vitro (Walls et al., 1996; Gleave et al., 1998; Melchior et al., 1999). This increase was found at the transcriptional level as well (Gleave et al., 1998). However, there are some discrepancies in the role of butyrate on PSA expression. In the experiments performed by Ellerhorst et al. (1999), butyrate caused a transient decrease in the amount of PSA protein to levels that were undetectable by immunoblotting. These results are at odds with those of other investigators who have shown that exposure of LNCaP cells to butyrate leads to increased amounts of secreted PSA. Since the decrease shown by Ellerhorst et al. (1999) was transient with the intracellular levels of PSA protein returning to near basal levels within 48 h of exposure, this discrepancy may be due to differences in experimental design. It appears that, at least in vitro, butyrate may increase PSA expression at several levels. The short half-life of butyrate has negated evaluation of this pharmacological agent in vivo and has led to the evaluation of butyrate analogs that have more favorable half-lives and/or bioavailability.

2. *Phenylbutyrate.* Phenylbutyrate (PB) is a prodrug for PA and is, reportedly, more potent (Carducci et al., 1996). In vitro, PB had effects on LNCaP cell proliferation and PSA secretion similar to those observed with PA (Walls et al., 1996; Melchior et al., 1999). Both agents inhibited cell proliferation by approximately 60% at 5 mM after 5 or 6 days in culture while inducing an increase in PSA secretion. However, this concentration is probably not clinically achievable based on the clinically achievable ranges reported for PA, ranges of 200 to 300 $\mu\text{g/ml}$ or approximately 1 to 2 mM (Thibault et al., 1994, 1995). The induction of PSA secretion by 2.5 mM PB was about 2-fold after 5 days of exposure. This effect was only observed, however, in the presence of andro-

gens. When cells were grown in charcoal-stripped serum (CSS), PSA secretion was only slightly increased. As with PA, PB-treated cells demonstrated an increase in the PSA transcript level suggesting a similar mechanism of regulation. Melchior et al. (1999) also showed that PB induced cell cycle arrest in G_0/G_1 and caused a 6-fold increase in apoptosis in both androgen-depleted and androgen-containing media. Upon cell cycle arrest, an induction of p21^{WAF1/CIP1}, a regulator of the G_1/S phase checkpoint (El-Deiry et al., 1994; Bissonette and Hunting, 1998), was noted. PA and butyrate have shown a similar induction of p21^{WAF1/CIP1} (Gorospe et al., 1996; Archer et al., 1998; Yamamoto et al., 1998).

The effects of PB on tumor growth were studied using xenografts of both LNCaP and LuCaP 23.1, another androgen-sensitive PSA-producing xenograft model (Ellis et al., 1996) with and without surgical castration (Melchior et al., 1999). Both castration and PB treatment significantly inhibited tumor growth while the combination of castration and PB was synergistic. The LuCaP 23.1 xenograft was much more responsive to all treatments than LNCaP. The effects of PB on serum PSA levels were variable. However, in LuCaP 23.1 xenografts, PB alone resulted in increased PSA levels while castration or castration plus PB caused a decrease. The median PSA levels in the castration plus PB-treated animals were higher than castration alone. The apoptotic index in LNCaP xenografts appeared to be slightly increased in animals with castration plus PB treatment. As in the studies by Walls et al. (1996), there is no mention of the differentiation status of any of the tumor xenografts. The results of clinical trials with PB have not been reported so it is unclear what the effects of this compound are in patients.

3. *Isobutyramide.* Isobutyramide is an orally bioavailable analog of butyrate with a longer half-life (Gleave et al., 1998). This compound was tested for its effects on LNCaP cell growth and morphology in vitro (Gleave et al., 1998). Isobutyramide caused a potent inhibition of cell proliferation, cell cycle arrest in G_1/G_0 , and a change in cellular morphology similar to what was observed for PB (Melchior et al., 1999). As with PB, isobutyramide-treated animals showed an inhibition of tumor growth compared with controls, and PSA mRNA levels in LNCaP tumors increased 2- to 3-fold in response to isobutyramide exposure. The serum PSA levels rose as the tumor progressed; however, the treated mice had serum PSA levels that were less than (>50%) the control mice. Thus, the secreted PSA levels did not increase in a manner consistent with what was observed for the RNA levels in the in vivo scenario. However, neither the changes in the serum PSA measured nor the PSA mRNA increase observed by Northern blotting were normalized to tumor volume. Lack of normalization may account for some of the discrepancy. The effect of isobutyramide on secreted PSA in vitro was not tested. This finding suggests that isobutyramide regulates PSA ex-

pression at a post-transcriptional level in addition to its effects at the transcriptional level. It appears that the mechanism of action of isobutyramide may be very similar to that of butyrate, PA, and PB (Walls et al., 1996; Gleave et al., 1998; Ellerhorst et al., 1999; Melchior et al., 1999) suggesting that the use of PSA as a surrogate marker is questionable with these compounds.

F. Vitamin D₃ and Synthetic Vitamin D Analogs

1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] is the active metabolite of vitamin D and an important regulator of calcium and phosphate homeostasis in the body (Holick, 1991; Feldman et al., 1996). It has been shown to have antiproliferative and differentiating effects on prostate cancer cell lines (Skowronski et al., 1993; Peehl et al., 1994; Miller et al., 1995; Hsieh et al., 1996). Those cell lines that express the highest levels of the vitamin D receptor are those that have the greatest antiproliferative response (Hedlund et al., 1997). Exposure of LNCaP cells to 100 nM 1,25(OH)₂D₃ for 48 h resulted in a 2-fold increase in both secreted and intracellular PSA (Hsieh et al., 1996; Walls et al., 1996). This same group demonstrated that increased PSA expression was accompanied by a parallel increase in AR expression, suggesting that 1,25(OH)₂D₃ may have a direct effect on AR transcription. Alternatively, the investigators speculated that 1,25(OH)₂D₃ may facilitate translocation of the AR from the cytoplasm to the nucleus either singly or through the cooperative action of another receptor such as the vitamin D₃ receptor.

Zhao et al. (1997) also reported that 1,25(OH)₂D₃ induced PSA secretion in a dose-dependent manner in LNCaP cells. These authors further reported that dihydrotestosterone (DHT) and 1,25(OH)₂D₃ were synergistic in the induction of PSA. When LNCaP cells were grown in medium supplemented with fetal bovine serum (FBS), the combination of these two hormones induced PSA secretion 22-fold compared with 5-fold for either hormone alone. When the cells were grown in medium containing CSS, FBS from which endogenous androgens and many growth factors are depleted, the combination led to a 51-fold increase in PSA secretion. In this same medium, DHT stimulated PSA secretion 11-fold and 1,25(OH)₂D₃ did not affect PSA secretion at all. The antiproliferative effects of 1,25(OH)₂D₃ on LNCaP cells were abrogated in the CSS medium; this inhibition could be reversed by the addition of 1 nM DHT to the medium. These observations led the authors to propose that the actions of 1,25(OH)₂D₃ are mediated by the AR. However, the questions still remain about whether cooperation occurs between the AR and the vitamin D₃ receptor or whether there is a direct effect of 1,25(OH)₂D₃ on the AR or its transcription.

Due to the hypercalcemic effect of 1,25(OH)₂D₃, it is not widely used as a chemotherapeutic agent. This side effect in conjunction with the potential therapeutic benefits of vitamin D led to the synthesis of several vitamin

D analogs that retain the antiproliferative and differentiating properties of the parent compound without the effects on calcium homeostasis. Several synthetic vitamin D analogs have been tested in human prostate cell lines; the results suggest that they have similar effects to 1,25(OH)₂D₃ (Peehl et al., 1994; Schwartz et al., 1994, 1995; Skowronski et al., 1995). Hedlund et al. (1997) evaluated 13 analogs in ALVA-31 and LNCaP, human prostate cell lines with high constitutive expression of vitamin D receptors. Three analogs were more potent at inhibiting cell proliferation in both ALVA-31 and LNCaP when compared with 1,25(OH)₂D₃ and induced PSA secretion in LNCaP. These results suggested that the effects of 1,25(OH)₂D₃ and its analogs are mediated through the vitamin D receptor. No experiments were performed to determine whether the analogs were synergistic with androgens as was reported for DHT and 1,25(OH)₂D₃ (Zhao et al., 1997). The clinical usefulness of vitamin D analogs and what their effect on PSA is in patients with AIPC has yet to be determined.

V. Agents That Down-Regulate Prostate-Specific Antigen

A. Gallium Nitrate

Gallium is a naturally occurring group IIIA heavy metal that has shown antitumor activity (Keller et al., 1986; Warrell et al., 1987; Chitambar et al., 1991; Seidman and Scher, 1991; Todd and Fitton, 1991; Baselga et al., 1993). In animal models, gallium nitrate showed potent cytotoxic activity with minimal toxicity (Hart et al., 1971). In both the PC-3 and LNCaP cell lines, we have shown that gallium nitrate has a concentration-dependent cytotoxicity (Dixon et al., 1997). After 5 days of treatment with 1.3 to 12 μ g/ml gallium nitrate, 34.2 to 66.5% of the LNCaP cells were viable. This decrease in cell number was accompanied by a 31.9- to 36.6-fold decrease in secreted PSA per cell that was also concentration-dependent (S. C. Dixon, unpublished results).

There have been several hypotheses concerning the mechanism of action of gallium nitrate (Larson et al., 1980; Chitambar et al., 1991; Berggren et al., 1993). Larson et al. (1980) have shown that gallium complexes with transferrin and is taken up by tumor cells via the transferrin receptor and appears to interfere with enzymes that utilize iron as a cofactor. In particular, the action of ribonucleotide reductase is inhibited leading to a decrease in the pool of deoxyribonucleotides (Chitambar et al., 1991). In addition, gallium nitrate has been shown to inhibit tyrosine phosphatase activity (Berggren et al., 1993). This observation suggests that some of the effects observed with gallium nitrate may be through its interference with cellular signal transduction mechanisms.

Scher et al. (1987) have published the results of a clinical trial in patients with AIPC treated with gallium nitrate. Two of 23 patients had a partial response as

evidenced by a reduction of soft tissue disease, whereas seven were reported to have a reduction in bone pain. However, since this trial was completed before the widespread use of PSA as a surrogate marker, no data is available regarding its changes in these patients. Our group has also completed a phase II clinical trial of gallium nitrate in patients with AIPC (Senderowicz et al., 1999). In this study, gallium nitrate had modest clinical antitumor activity based on PSA responses. One patient had a partial response based on a greater than 75% decrease in serum PSA levels that lasted for 4 months, whereas three other patients demonstrated stable disease based on PSA. The decreases in PSA observed in these patients were transient and occurred during the administration of gallium. Following discontinuation of treatment, PSA rose before the next cycle. These observations suggest that the decreased PSA were at least partially due to an inhibitory effect of gallium on PSA secretion.

B. Troglitazone

Troglitazone is a thiazolidinedione derivative that is currently used as a therapeutic agent for insulin-resistant diabetes mellitus (Kubota et al., 1998). Thiazolidinediones are specific ligands for the nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) (Forman et al., 1995; Lehmann et al., 1995). This receptor is highly expressed in adipose tissue and is hypothesized to play a central role in adipocyte differentiation (Tontonoz et al., 1994, 1997); terminal differentiation was also induced in breast cancer cells (Mueller et al., 1998). These studies suggested that thiazolidinediones might have differentiation and antiproliferative properties in other cell types as well. Kubota et al. (1998) evaluated the *in vitro* effects of troglitazone on the PPAR γ expressing PC-3, DU145, and LNCaP cell lines, as well as *in vivo* in PC-3 xenografts (Kubota et al., 1998). Under the growth conditions used, troglitazone inhibited the growth of PC-3 cells by greater than 70%, but by less than 80% in DU145 and LNCaP. Troglitazone also was effective in suppressing the growth of PC-3 xenografts *in vivo*. Exposure of LNCaP cells to troglitazone showed an approximate 50% reduction in secreted PSA per 10^5 cells.

The PPAR γ can form heterodimers with the retinoic acid receptor RXR α that binds 9-*cis*-retinoic acid (9-*cis*-RA) and the synthetic ligand LG100268 (Dreyer et al., 1992; Kliewer et al., 1992, 1994). In the presence of both ligands, the receptor complex can bind to DNA resulting in the regulation of target genes. Kubota et al. (1998) tested whether simultaneous exposure of prostate cell lines to troglitazone and LG100268 produced an additive or synergistic effect on cell proliferation. They showed a slight additive effect on inhibition of growth of PC-3 but not LNCaP or DU145 cells. None of the cell lines showed an accumulation of cells in the G₁/G₀ phase of the cell cycle with either drug alone or together. The effect of

LG100268 or the combination of troglitazone and LG100268 on PSA secretion was not evaluated in these experiments. These experiments suggest that there is some sort of block in the promotion of terminal differentiation in these cell lines. It has been suggested that the enzyme mitogen-activated protein kinase, which is elevated in some tumors, can phosphorylate the PPAR γ leading to reduced transcriptional activity and a loss of its differentiation properties (Hu et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997; Sivamaran et al., 1997). This has been shown to be the case in the metastatic breast cancer cell line 21-MT by Mueller et al. (1998). When this cell line was treated with an inhibitor of mitogen-activated protein kinase kinase, there was an increase in the unphosphorylated form of PPAR γ and an increase in its transcriptional activity. In the presence of troglitazone alone, there was a minimal response.

C. Carboxyamido-Triazole

Carboxyamido-triazole (CAI) interferes with calcium influx through its inhibition of nonvoltage-gated calcium channels (Felder et al., 1991; Hupe et al., 1991; Cole and Kohn, 1994). CAI has been shown to be effective at suppressing proliferation, migration, and metastasis of several cell types (Kohn et al., 1992; Cole and Kohn, 1994). In addition, it has been shown to have antiangiogenic properties in several model systems (Kohn et al., 1995; Bauer et al., 2000). Wasilenko et al. (1996) demonstrated that CAI suppressed the growth of several prostate cell lines in a dose-dependent manner. The prostate cell lines varied in their sensitivity to CAI; PPC-1 was the most sensitive and PC-3 the least. LNCaP and DU145 exhibited comparable sensitivity. The IC₅₀ values for these cell lines ranged from 10 to 30 μ M. At clinically achievable concentrations of CAI (1–10 μ M), there was approximately a 1.7- to 2.5-fold reduction in PSA secretion from LNCaP cells.

In a phase II clinical trial, it was concluded that CAI had no clinical activity in AIPC (Bauer et al., 1999). Fourteen of 15 patients could not be evaluated, but all had progressive disease within approximately 2 months following enrollment. Although there was no clinical activity, 9 of the 15 patients had a transient PSA decrease of 14.3% from baseline. Thus, both the *in vitro* studies and a clinical trial showed a CAI mediated decline in PSA. However, in the clinical trial, this decrease in PSA did not correspond with a decreased burden of disease.

D. Finasteride

Finasteride is a competitive inhibitor of 5 α -reductase, the enzyme responsible for the conversion of testosterone to DHT (Sudduth and Koronkowski, 1993). DHT has a higher affinity for the AR than testosterone and is the predominant ligand for the AR *in vivo* (Kumar et al., 1999). The role of androgens in the regulation of prostate growth has stimulated the extensive use of finasteride in

the treatment of benign prostatic hypertrophy (Huggins and Hodges, 1941; Sudduth and Koronkowski, 1993). It is also currently being tested as a chemopreventive agent for prostate cancer (Thompson et al., 1997).

Two groups have shown that finasteride can inhibit the in vitro growth of LNCaP and, to a lesser degree, PC-3 and DU145 cell lines (Bologna et al., 1995; Kreis et al., 1997). Wang et al. (1997) extended the study on the effects of finasteride on LNCaP cells by looking at PSA regulation. They found that inhibition of PSA secretion from LNCaP cells was maximal with 25 μM finasteride. At this concentration, PSA secretion was decreased by 56% without a significant loss in cell viability. Decreased amounts of PSA were also reflected at the intracellular protein and RNA levels in a time- and dose-dependent fashion. These results suggested that finasteride regulated PSA gene expression transcriptionally. Previous work showed that the transcriptional regulation of the PSA gene by androgens was modulated by the SRBC (Luke and Coffey, 1994). When transcription of the PSA gene is stimulated, a complex of proteins responsible for the transcriptional transactivation of PSA bind to this site. Wang et al. (1997) showed that treatment with 25 μM finasteride resulted in decreased binding of complexes at the SRBC that directly correlated with a decrease in PSA secretion and expression. These investigators also showed that the AR was part of the binding complex. When the nuclear extracts were depleted of AR, there was a loss of binding complexes at the SRBC. Therefore, the decrease in PSA expression by finasteride was mediated at the transcriptional level through a nuclear protein complex that involved the AR.

Finasteride has been evaluated in a randomized placebo controlled study in patients with untreated stage D prostate cancer (Presti et al., 1992). In this 12-week study, it was found that there was a significant decrease in PSA levels in those patients taking finasteride compared with those on placebo. The finasteride-treated patients had a 15.1% decrease in PSA, whereas those on placebo had an 11.7% increase ($p < 0.05$). Both groups had comparable decreases in prostatic volume. However, changes in prostatic volume usually occur slowly, with maximal effects requiring 26 weeks (Gormley et al., 1992). Thus, it is possible that a longer study duration might have yielded a significant change in this parameter. In another study, Cote et al. (1998) found that the PSA levels of men treated with finasteride decreased by 48%, whereas in men given a placebo, it was unchanged after 12 months. All men had elevated PSA and negative sextant biopsies at the initiation of the study. In addition, they found that 30% of the finasteride-treated men developed prostate cancer during the study compared with only 4% of the men in the placebo group ($p = 0.025$). There was no significant change in hyperplastic or prostatic intraepithelial neoplastic lesions between the two groups. Thus, although finasteride appears to have a

down-regulatory effect on PSA secretion, its effect on the chemoprevention of prostate cancer is controversial.

E. Leuprolide Acetate

Leuprolide acetate is a luteinizing hormone-releasing hormone analog (LHRHa) that is used to block the secretion of androgens from the adrenal gland and is commonly included in the medical castration regimen for prostate cancer (Auclerc et al., 2000). Under controlled growth conditions, Sica et al. (1999) reported that this agent was ineffective at inhibiting growth of LNCaP, PC-3, and DU145 cell lines in vitro. However, it was capable of blocking androgen-stimulated growth in LNCaP cells and epidermal growth factor (EGF)-induced growth in PC-3. Although leuprolide acetate had no effect on unstimulated cell growth, it significantly down-regulated the expression of PSA mRNA. In LNCaP cells, leuprolide acetate alone could reduce PSA mRNA levels to undetectable amounts. In the presence of DHT, leuprolide acetate reduced PSA gene expression to levels observed in untreated cells. They also found that in PC-3 cells induced to produce PSA by treatment with EGF, as determined by reverse transcription-polymerase chain reaction, that leuprolide acetate could also block the EGF-induced expression of this gene. These results not only implicate the AR in the control of PSA gene transcription, but they also show that other growth factors exert regulatory controls on this gene. In agreement with previous studies, these results suggest that there is cross-talk between the cellular responses mediated by the AR and those of other growth factors (Culig et al., 1994).

F. PC-SPES

PC-SPES is a commercially available herbal preparation that consists of one American and seven Chinese herbs: isatis, Panax-pseudo ginseng, chrysanthemum, licorice, saw palmetto, skullcap, *Ganoderma ludidium*, and *Rabdosia rubescens* (Fan and Wang, 1995). It has been shown to have potent estrogenic activity both in vitro and in vivo. A 1:200 dilution of an ethanolic extract produced equivalent effects to 1 nM estradiol in a transcriptional activation assay in yeast (DiPaola et al., 1998). When female CD1 mice were treated with a suspension of PC-SPES, uterine weight was significantly increased compared with controls (DiPaola et al., 1998). Using a high-performance liquid chromatography "standardized" ethanolic extract of PC-SPES, Hsieh et al. (1998) showed that LNCaP cell growth was inhibited in a time- and concentration-dependent manner. A similar finding using both LNCaP and PC-3 cells was reported by Halicka et al. (1997). Inhibition of cell growth was accompanied by increased apoptosis (Halicka et al., 1997) and a decrease in proliferating cell nuclear antigen (PCNA), which is used as an indicator of mitotic index (Hsieh et al., 1997). The amount of both secreted and intracellular PSA was reduced in LNCaP cells (Hsieh et

al., 1997). Secreted PSA was reduced by 60 to 70%, whereas intracellular PSA decreased by only 20 to 40%. The secreted PSA was not normalized to account for differences in cell growth that may account for the difference observed with the intracellular PSA decreases. These investigators also found that the decrease in intracellular PSA was paralleled by decreased amounts of AR protein. These results point to both post-transcriptional and post-translational regulation of PSA by PC-SPES. Since protein levels of the AR are also effected by treatment of cells with PC-SPES, control may also be mediated at a transcriptional level. In another study performed on the B cell-derived cell line, Mutu I, the proto-oncogene *bcl-6* was down-regulated by 60 to 72% by PC-SPES (Hsieh et al., 1998). Since *bcl-6* has been proposed to act as a transcriptional repressor (Deweindt et al., 1995; Chang et al., 1996), it may be at least partially responsible for the decreases observed in both PSA and AR levels. Alternatively, it is possible that *bcl-6* may in some way modulate AR activity leading to the down-regulation of PSA in prostate cell lines. Confirmation of these hypotheses requires further investigation.

The use of PC-SPES as a dietary supplement in men with prostate cancer has been evaluated in two clinical trials. The side effects were limited to breast tenderness and one incident of superficial venous thrombosis (DiPaola et al., 1998; Pfeifer et al., 2000). Pfeifer et al. (2000) reported a reduction in patient perceived pain. Both studies reported reductions in serum PSA levels in all patients who received PC-SPES. However, within a few weeks of discontinuing PC-SPES, PSA levels began to rise. Neither study reported any changes in prostatic growth. Thus, although the use of PC-SPES may provide some clinical benefit to patients with prostate cancer, it may confound the interpretation of other concurrent therapies through its effects on PSA regulation.

G. Suramin

Suramin is a polysulfonated naphthylurea that has shown clinical activity in metastatic prostate cancer (Dawson et al., 1997), although a FDA advisory committee did not ultimately recommend it for approval. Suramin has been shown to inhibit the activity of several enzymes including reverse transcriptase (De Clercq, 1979) and protein kinase C (Hensey et al., 1989). It is known to inhibit the binding of growth factors and cytokines to their receptors (De Clercq, 1979; Hosang, 1985; Coffey et al., 1987; Moscatelli and Quarto, 1989; Fantini et al., 1990; Kim et al., 1991; Pienta et al., 1991; Strassman et al., 1993). It interferes with cell motility and metastasis (Kim et al., 1991; Pienta et al., 1991; Ellis and Dano, 1993), induces cell differentiation (Fantini et al., 1990) and is antiangiogenic (Gagliardi et al., 1992). The clinical benefit and antitumor activity of suramin therefore could be due to any of a number of its *in vitro* activities.

Suramin exhibits antitumor activity against human-derived prostate cancer cell lines (Yamazaki et al., 1984; La Rocca et al., 1991). Thalmann et al. (1996) studied the effect of suramin on the growth of the LNCaP cell line and its androgen-independent subline C4-2. They found that suramin had no effect on the growth of either androgen-independent C4-2 xenografts or of C4-2 cells *in vitro*, whereas the growth of androgen-dependent LNCaP cells in culture was transiently inhibited. The effect of suramin on the growth of LNCaP as a xenograft was not ascertained. Experiments performed by Arah et al. (1999) demonstrated that *in vitro*, suramin inhibited cell growth in LNCaP by 50 to 82% and in PC-3 cells by 13 to 38%.

Although no growth inhibition of C4-2 xenografts was observed, PSA secretion was significantly inhibited in the mice carrying C4-2 xenografts. In addition, the steady state amounts of PSA mRNA were reduced in both C4-2 and LNCaP *in vitro* (Thalmann et al., 1996). Thus, it appears that suramin may alter PSA expression apart from its inhibitory effect on tumor growth. In two other independent experiments, it was shown that suramin either caused a slight decrease in PSA secretion per cell from LNCaP cells that was not significant (Walls et al., 1996) or had no effect on PSA secretion (Arah et al., 1999). Discrepancies between these experiments are most likely due to differences in experimental design.

In two trials using suramin in men with hormone-refractory prostate cancer, it was found that 85 of 103 patients had some decrease in serum PSA levels after 4 weeks on suramin (Eisenberger et al., 1993, 1999; Sridhara et al., 1995). These men did not receive flutamide or leuprolide. They did receive hydrocortisone, but Thalmann et al. (1996) have reported that this agent has no effect on PSA secretion from LNCaP cells. Based on the findings from mice bearing C4-2 xenografts, it is likely that suramin is partially responsible for the PSA declines observed in these patients.

There are several explanations as to how suramin might regulate PSA expression. Some of the effects might be mediated through the AR, as has been observed for other pharmacological agents. Suramin has been proposed to interfere with the synthesis of adrenal androgen synthesis either through inhibition of enzymes involved in this metabolic pathway, or by direct toxic effects on the adrenal gland (Stein et al., 1986, 1989). However, the reduction in PSA expression was observed in serum-free growth conditions as well as in 5% serum. This result demonstrates that some of the effects of suramin are not mediated through the AR but must utilize another mechanism, such as interference with the action of growth factors or possibly through inhibition of a protein kinase C-mediated pathway. Finally, although suramin appears to have a regulatory effect on PSA expression, the means by which this effect occurs are complicated and not well understood.

H. Flavopiridol

Flavopiridol (L86-8275) is a flavone derivative that inhibits cyclin-dependent kinases, thereby blocking progression through the cell cycle and leading to cell death (Kaur et al., 1992; Worland et al., 1993). Flavopiridol at high concentrations, 400 to 800 nM, has been shown to be cytotoxic to LNCaP cells (S. C. Dixon, unpublished data). However, these concentrations are not clinically achievable. In the clinically achievable concentration range of 50 to 100 nM, flavopiridol significantly inhibited the growth of LNCaP cells. Flavopiridol at 10 nM had little effect on cell growth. In addition to a high degree of cytotoxicity, flavopiridol potently induced apoptosis within 24 h of exposure. The amount of cell death induced by 400 to 800 nM flavopiridol made measurement of PSA unreliable after 48 h of exposure. However, there did appear to be some down-regulation within the first 48 h at these concentrations. In the 10 to 100 nM concentration range, secreted PSA was not affected. Concentrations between 100 and 400 nM were not tested.

I. Estramustine Phosphate and Its Metabolites

Estramustine phosphate is a conjugate of β -estradiol and nor-nitrogen mustard that is metabolically activated in vivo (Tew et al., 1983). Its metabolites that include estramustine, estromustine, estrone, and β -estradiol cause disassembly of microtubules and inhibit their de novo formation (Friden et al., 1987; Stearns and Tew, 1988; Tew and Stearns, 1989; Benson and Hartley-Asp, 1990; Dahllhof et al., 1993). This results in mitotic arrest during metaphase and cell death (Hartley-Asp, 1984; Hansenson et al., 1988; Tew and Stearns, 1989; Benson and Hartley-Asp, 1990; Kreis et al., 1997; Arah et al., 1999). Wang et al. (1998) showed that estramustine metabolites, but not estramustine phosphate itself, can bind with various affinities and in a concentration-dependent manner to the mutant AR found in LNCaP cells or to HeLa cells transfected with a mutant AR. These characteristics have led to the widespread use of estramustine phosphate, either alone or in combination with other chemotherapeutic agents, in the treatment of hormone-refractory prostate cancer.

Estramustine has been shown to be cytotoxic to prostate cancer cell lines (Hartley-Asp and Gunnarsson, 1982; Hartley-Asp, 1984; Hansenson et al., 1988; Kreis et al., 1997; Arah et al., 1999). Arah et al. (1999) showed that estramustine comparably decreased both LNCaP and PC-3 cell growth in a concentration-dependent manner by 28 to 84%. In addition, they showed that estramustine caused a 53 to 90% concentration-dependent decrease in PSA secretion in LNCaP cells. Wang et al. (1998) demonstrated that estramustine significantly down-regulates PSA mRNA as well. The amount of PSA transcripts was decreased by 56 and 90% by 5 and 10 μ M estramustine, respectively. Comparison of the re-

sults obtained by these two groups suggests that estramustine regulates PSA gene expression at a transcriptional level. Given that estramustine can bind significantly to the mutant AR in LNCaP cells ($EC_{50} = 3.13 \pm 0.31 \mu$ M) (Wang et al., 1998), it is probable that the transcriptional regulation of PSA by estramustine is mediated through the AR. However, caution should be taken when trying to extend these results to a normal, wild-type AR.

Significant decreases in serum PSA levels in patients with hormone-refractory prostate cancer have not been observed in a phase I clinical trial using estramustine as a single agent (Haas et al., 1998). The objective response rate in this trial was also minimal (<20%). The results of clinical trials using estramustine in combination with other chemotherapeutic agents such as docetaxel, vinblastine, and etoposide have been more encouraging (Hudes et al., 1992; Pienta et al., 1994; Attivissimo et al., 1996; Colleoni et al., 1997; Cruciani and Turolla, 1998). In these trials, PSA declines of >50% were often observed. The decreases in PSA observed may have been due to the regulatory effects of the other agents used or to the combination of the agents. Both etoposide and vinblastine have proven to be too highly cytotoxic for their effects on PSA secretion in LNCaP cells to be determined (Arah et al., 1999).

J. Resveratrol

Resveratrol is a phytoalexin found in many dietary plants, including grapes and peanuts, that can inhibit all stages of malignant transformation: initiation, promotion, and progression (Jang et al., 1997). It also has been shown to inhibit the growth of hormone-sensitive and -refractory breast cancer cell lines (Mgbonyebe et al., 1998). Resveratrol is a potent antioxidant and can inhibit ribonucleotide reductase (Fontecase et al., 1998), DNA polymerase (Sun et al., 1998), and cyclooxygenase-1 and -2 (Jang et al., 1997; Subbaramaiah et al., 1998).

Recently, the effects of resveratrol were studied in human prostate cell lines (Hsieh and Wu, 1999; Mitchell et al., 1999). Both groups reported that resveratrol caused a significant decrease in cell proliferation. Hsieh and Wu (1999) reported, however, that the growth of the androgen-dependent LNCaP cell line was suppressed to a greater extent than that of the androgen-independent PC-3, DU145 or JCA-1 cell lines. In addition they reported that in the androgen-independent cell lines, there was a partial block of the G₁ to S phase transition. This block was not evident in the LNCaP cell line. However, LNCaP cells did show a higher percentage of apoptotic cells in response to resveratrol than did the androgen-independent cell lines. Both groups also demonstrated that PSA secretion was down-regulated in response to resveratrol; Hsieh and Wu (1999) extended this observation to the intracellular protein level (Mitchell et al., 1999). Controversy arises as to how resveratrol might be

affecting PSA expression. Hsieh and Wu (1999) showed that there was no decrease in AR binding, nor AR protein expression, after 4 days of treatment with 0.25 to 25 nM resveratrol. They concluded that the effects of resveratrol were not mediated through the AR. On the other hand, Mitchell and colleagues (1999) found that exposure of LNCaP cells to 50 to 150 μ M resveratrol significantly reduced AR protein levels, and down-regulated the expression of three other androgen-regulated genes: human kallikrein 2 (hKLK2), ARA70, and p21^{WAF1/CIP1}. In addition, they showed that the presence of resveratrol abrogated transcription from reporter gene constructs containing either the androgen-regulated PSA promoter or multiple copies of the androgen response element.

The conflicting nature of these results makes it difficult to determine whether resveratrol exerts its effects in an androgen-dependent or -independent manner. Differences in results between these two groups could be attributed to the concentrations of resveratrol used. Mitchell et al. (1999) used concentrations that were much higher, and may not be clinically achievable, than those of Hsieh and Wu (1999). Or some other experimental variables such as cell passage, culture conditions, etc., may be responsible. It may be that at lower concentrations of resveratrol, the principal mechanism of action is by the regulation of the abundance of AR accessory proteins, such as ARA70, or by inhibiting the binding of the AR to its DNA response elements. Effects on AR abundance may only be evident at high concentrations of resveratrol.

VI. Agents That Have a Dual Effect On Prostate-Specific Antigen

Retinoids are analogs of vitamin A. There are a number of natural and synthetic retinoids including retinol, 9-*cis*-RA, 13-*cis*-RA, all-*trans*-RA, and *N*-(4-hydroxyphenyl)retinamide (4-HPR). These compounds have been shown to block the phenotypic expression of cancer cells in both human and animal models regardless of the promoting factors (Lasnitzki and Goodman, 1974; Sporn et al., 1976; Chopra and Wilkoff, 1979; Moon et al., 1983). Thus, retinoids can inhibit the proliferation of both normal and cancerous prostate cells and are capable of inducing differentiation (Lippman et al., 1987; Pollard et al., 1991). Carter et al. (1990) have reported that the risk of prostate cancer decreases with the uptake of high doses of vitamin A. The regulation of retinoids is complex, and their effects are mediated through either the retinoid acid receptor α (RAR α) or the retinoid X receptor α (RXR α) (Petrovich et al., 1987; Brand et al., 1988; Mangelsdorf et al., 1990; Blumberg et al., 1992; Levin et al., 1992). This complexity doubtless leads to the disparate effects of these agents on PSA regulation.

A. Retinol, 9-*cis*-Retinoic Acid, and 13-*cis*-Retinoic Acid

Young and colleagues (1994) looked at the effects of retinol and retinoic acid on LNCaP cells both alone and

in the presence of androgenic stimulation. They found that both retinol and retinoic acid (form unspecified) inhibited the growth of androgen-stimulated LNCaP cells. Retinol was a less efficient inhibitor than retinoic acid as was expected from a previous study showing that retinol is less potent than retinoic acid (Romjin et al., 1988). Young et al. (1994) found that the growth inhibition was accompanied by a down-regulation of AR protein but did not effect ligand binding. When they looked at PSA and hKLK2, they found that both genes were down-regulated similar to the AR. Down-regulation of PSA and hKLK2 was found for secreted and intracellular protein and for mRNA suggesting that the down-regulation of the AR by retinoic acid was responsible. However, it is interesting that the loss of AR protein and, therefore, a loss of transactivation potential may not be the primary cause of the decreased PSA and hKLK2 expression. In addition, the down-regulation of PSA mRNA reported by these authors appears to be minimal when compared visually to the control gene, glyceraldehyde-3-phosphate dehydrogenase. The Northern analysis was not quantitated in any way, compromising the interpretation of these data. The maximal repression observed for intracellular PSA and hKLK2 protein occurred at 24 h after exposure to retinoic acid, whereas the maximal repression of AR protein did not occur until 36 h. The investigators interpreted these data to suggest that other factors regulated by retinoic acid might influence the transactivation functions of the AR or the transcription of PSA and hKLK2. However, since PSA mRNA does not seem to be significantly down-regulated, it may be more likely that PSA is down-regulated during some post-transcriptional event that may not rely on the AR, leading to the decreased intracellular and secreted PSA.

In another study, 1 μ M 13-*cis*-RA down-regulated secretion of PSA from LNCaP cells by greater than 3-fold (Dahiya et al., 1994). Both intracellular PSA protein and PSA mRNA were down-regulated similarly to secreted PSA, suggesting that 13-*cis*-RA exerted its effects either at the transcriptional or post-transcriptional level. At the same concentration of 13-*cis*-RA, the growth of LNCaP cells was inhibited. DNA synthesis was decreased by 2-fold while doubling time was doubled. In addition, this concentration of 13-*cis*-RA showed little cytotoxicity (>95% of the cells were viable), although cell morphology also changed. Whether this change is toward a more differentiated phenotype is difficult to conclude since there are no defined parameters to describe a differentiated prostate cell.

B. All-*trans*-Retinoic Acid

The demonstration that PA induced the secretion of PSA from LNCaP cells prompted Walls et al. (1999) to determine the effects of other well known differentiation agents in this system. When LNCaP cells were exposed to 3 μ M all-*trans*-RA, PSA secretion was induced ap-

TABLE 1
Regulation of PSA and activity of selected therapeutic targets

Pharmacological Agent	Effect in vitro	Effect in Animal Model	Effect in Men with AIPC	Reference
Thalidomide	Increased PSA secretion; slight growth inhibition	N.A.	N.A.	Dixon et al., 1999
TNP-470, AGM-1883	Increased PSA secretion; growth inhibition	N.A.	Reversible increases in PSA	Horti et al., 1999; Logothetis et al., 2000; Sartor, 1995
GM-CSF	Increased PSA secretion; growth inhibition	N.A.	Oscillations in PSA or decreased PSA on therapy	Small et al., 1999
Phenylacetate	Increased PSA secretion; cytostatic	Increase in the number of PSA-producing cells; fewer mitotic figures than controls	Stable or decreased PSA	Thibault et al., 1994; Walls et al., 1996
Butyrate	Increased PSA secretion; growth inhibition	N.A.	N.A.	Ellerhorst et al., 1999; Walls et al., 1996
Phenylbutyrate	Increased PSA secretion; growth inhibition	Increased PSA secretion; growth inhibition	N.A.	Melchior et al., 1999; Walls et al., 1996
Isobutyramide	Increased PSA secretion; growth inhibition	Increased PSA secretion; growth inhibition	N.A.	Gleave et al., 1998
Vitamin D and its analogs	Increased PSA secretion; growth inhibition	N.A.	N.A.	Hedlund et al., 1997; Hsieh et al., 1996; Peehl et al., 1994; Schwartz et al., 1994; Skowronski et al., 1993; Zhao et al., 1997
Gallium nitrate	Decreased PSA secretion; cytotoxic	N.A.	Transient decreases in PSA	Dixon et al., 1997; Scher et al., 1987; Senderowicz et al., 1999; S. C. Dixon, unpublished data
Troglitazone	Decreased PSA secretion; growth inhibition	Growth inhibition of PC-3 xenografts	N.A.	Kubota et al., 1998
CAI	Decreased PSA secretion; growth inhibition; cytotoxicity at high doses	N.A.	Decreases in PSA; no clinical activity	Bauer et al., 1999; Wasilenko et al., 1996
Finasteride	Decreased PSA secretion; growth inhibition	N.A.	Decreases in PSA; decreases in prostatic volume	Bologna et al., 1995; Kreis et al., 1997; Presti et al., 1992; Wang et al., 1997
Leuprolide acetate	Decreased PSA secretion; no effect on cell growth	N.A.	Decreases in PSA	Sica et al., 1999
PC-SPES	Decreased PSA secretion; growth inhibition	N.A.	Decreases in PSA	DiPaola et al., 1998; Halicka et al., 1997; Hsieh et al., 1997, 1998; Pfeifer et al., 2000
Suramin	Decreased or no effect on PSA secretion; growth inhibition	Decreases in PSA; no effect on growth of C4-2 xenografts	Decreases in PSA	Arah et al., 1999; Eisenberger et al., 1993, 1995; Sridhara et al., 1995; Thalmann et al., 1996; Walls et al., 1996
Flavopiridol	Decreased PSA secretion; growth inhibition; apoptosis induction	N.A.	Decreases in PSA	S. C. Dixon, unpublished data
Estramustine phosphate	Decreased PSA secretion; cytotoxic	N.A.	No significant decreases in PSA	Arah et al., 1999; Attivissimo et al., 1996; Colleoni et al., 1997; Cruciani and Turolla, 1998; Hansson et al., 1988; Hartley-Asp, 1984; Hudes et al., 1992; Kreis et al., 1997; Pienta et al., 1994; Wang et al., 1998
Resveratrol	Decreased PSA secretion; growth inhibition	N.A.	N.A.	Hsieh and Wu, 1997
Retinol, 9- <i>cis</i> -retinoic acid, 13- <i>cis</i> -retinoic acid	Decreased PSA secretion; growth inhibition	N.A.	N.A.	Dahiya et al., 1994; Lippman et al., 1987; Pollard et al., 1991; Young et al., 1994

All- <i>trans</i> -retinoic acid	Increased PSA secretion; growth inhibition	N.A.	Walls et al., 1996
4-HPR	Decreased PSA secretion; growth inhibition	N.A.	Hsieh and Wu, 1997; Igawa et al., 1997; Pienta et al., 1993, 1997; Pollard et al., 1991; Shen et al., 1999; Slawin et al., 1993 Davis and Sarkar, 2000
Genistein	Decreased or no effect on PSA secretion; growth inhibition	N.A.	Thalmann et al., 1996
Hydrocortisone	No effect on PSA secretion; growth inhibition	N.A.	M. Rudek, personal communication
COL-3	No effect on PSA secretion; growth inhibition	N.A.	S. C. Dixon, unpublished data
Prednisone	No effect on PSA secretion; growth inhibition	N.A.	Lim et al., 2000
Exisulind	No effect on PSA secretion; apoptotic induction	N.A.	M. V. Blagosklonny, personal communication
Dolastatin	No effect on PSA secretion; growth inhibition	N.A.	

N.A., not applicable.

proximately 2-fold. All-*trans*-RA binds to the RAR α and mediates its differentiating effects through this receptor (Kamei et al., 1994; Xue et al., 1996). Thus, in prostate cancer cells, it appears that all-*trans*-RA leads to differentiation and up-regulation of PSA secretion; this response is mediated through signal transduction events mediated by RAR α .

The effects on PSA levels observed with all-*trans*-RA are contrary to those observed for 9-*cis*-RA and 13-*cis*-RA. It is unclear what the differences are in the mechanism of action of these compounds in regard to PSA regulation. It is possible that the choice of which receptor is activated, RAR α or RXR α , and the cellular responses that each mediates may be responsible for the differences observed in the regulation on PSA by retinoids. As mentioned above, all-*trans*-RA utilizes the RAR α (Kamei et al., 1994; Xue et al., 1996). 13-*cis*-RA, on the other hand, is a ligand for the RXR α . Exposure of LNCaP cells to 13-*cis*-RA leads to up-regulation of the RXR α gene (Dahiya et al., 1994). 9-*cis*-RA has a higher affinity for RXR α but is also able to activate the RAR α (Levin et al., 1992). Alternatively, the presence or absence of specific cellular retinoic acid-binding proteins that modulate the cellular response to retinoids may play a role in PSA regulation (Dahiya et al., 1994).

C. N-(4-Hydroxyphenyl)retinamide

Although retinoids were efficacious as chemopreventive agents of prostate cancer in experimental models, their high degree of toxicity limited their usefulness in humans. To this end, synthetic analogs that were less toxic were derived. One such analog is 4-HPR. 4-HPR effectively suppressed tumor growth in both in vitro and in vivo model systems and inhibited invasiveness in vitro (Pollard et al., 1991; Pienta et al., 1993; Slawin et al., 1993; Hsieh and Wu, 1997; Igawa et al., 1997; Shen et al., 1999). When LNCaP cells were exposed to 4-HPR, there was a dose-dependent inhibition of cell growth (Hsieh and Wu, 1997; Shen et al., 1999). The growth of PC-3 cells was inhibited but to a lesser extent than LNCaP cells (Igawa et al., 1997; Shen et al., 1999). In addition to growth inhibition, both groups showed that 4-HPR caused a dramatic change in cellular morphology, arrest in the G₁ phase of the cell cycle, and induced apoptosis in LNCaP cells. Hsieh and Wu (1997) showed that PSA expression was down-regulated between 17 and 68% at 1 and 5 μ M 4-HPR, respectively, when LNCaP were grown in the presence of FBS (which contains androgen). Shen et al. (1999) reported that when LNCaP cells were grown without androgen, 4-HPR blocked androgen-induced PSA secretion by 50 to 80%. Down-regulation of PSA was observed at both the protein and mRNA levels (Hsieh and Wu, 1997; Shen et al., 1999). The amount of down-regulation of PSA mRNA was much less than reported for secreted PSA. This may be due to the lack of normalization of the secreted PSA to the reduced cell number in the presence of 4-HPR. Con-

comitant with the down-regulation of PSA protein, Hsieh and Wu (1997) demonstrated that the AR was down-regulated by 35 to 80%. Thus, it appears that down-regulation of PSA by 4-HPR is mediated through the AR.

4-HPR has been investigated in a phase II chemoprevention trial for prostate cancer (Pienta et al., 1997). There were several problems in the design and implementation of this trial. The most limiting problem was the small number of patients that remained on study that could be evaluated. At the end of 6 months, the PSA level of one patient had doubled and he was taken off study. In addition, all patients had negative prostate biopsies within 3 months of starting the trial. At the conclusion of the trial, 4 of the 16 patients that remained had positive biopsies. Pre- and post-study measurements of prostate gland volume were performed on different equipment and a comparison was not valid. From this trial, it is not possible to determine the *in vivo* effects of 4-HPR on PSA secretion. In addition, it appears that 4-HPR is ineffective in the prevention of prostate cancer; however, the small sample size compromised the power of statistical analyses. In addition, one of the trial's criteria was a PSA level of >4.0 ng/ml. Using this criteria, it is argued that the study design was not appropriate for chemoprevention, but for treatment, since men with a PSA > 4.0 ng/ml would be suspect for having prostate cancer. The evaluation of 4-HPR on PSA regulation in prostate cancer requires further investigation.

VII. Discussion

PSA is the one of the most widely used surrogate markers for disease progression and treatment response. Elevated levels of PSA are taken to be indicative of high tumor burden, evidence of disease progression, or indicating a lack of response to a particular therapeutic agent. Lower PSA levels are suggestive of a beneficial response or a decrease in tumor burden. However, the scientific literature clearly demonstrates that PSA expression and secretion are regulated by pharmacological agents. The expression of PSA and cellular proliferation are independently regulated functions in the prostate cancer cell (Cunha et al., 1987). This observation raises the possibility that regulation of PSA expression by pharmacological agents or their metabolites may occur independently of any effect on cell growth or proliferation. For the interpretation of clinical data based on serial PSA measurements to be valid, it is important to know the effects of a particular pharmacological agent on the regulation of PSA expression and secretion. If this action of the agent is not accounted for, it is possible that a potentially beneficial agent might be discontinued due to its ability to up-regulate PSA secretion, even though it may effectively abrogate tumor growth and/or metastasis. Alternatively, an agent may down-regulate PSA se-

cretion but have little or no effect on tumor growth, leading to the continuation of an ineffective therapy. Thus, noncytotoxic agents need to be prospectively evaluated for their effects on PSA secretion per cell number or cell mass.

A summary of the agents reviewed in this article and their effect on PSA expression/secretion is shown in Table 1. There appear to be two mechanisms by which a pharmacological agent may regulate PSA expression and secretion. The first is by means of an androgen-dependent mechanism. In this mechanism, the agent in question either alters the abundance and/or the function of the AR. The function of the AR may be changed through the availability of certain accessory proteins that interact with the AR, by changes in its phosphorylation status, or by inhibiting or accentuating its binding to its response element in the promoter of the PSA gene. Since the AR is responsible for the transcriptional transactivation of the PSA gene, any change in its status is likely to effect PSA expression in some way (Cleutjens et al., 1996). A review of the literature shows that there are several agents that apparently work in this manner including vitamin D and its analogs, retinoids, TNP-470 and its metabolite AGM-1883, GM-CSF, phenylbutyrate, finasteride, leuprolide acetate, PC-SPEs, estramustine, and flavopiridol.

The second mechanism by which an agent may regulate PSA expression is in an androgen-independent manner. In this mechanism, there is far more variation regarding the site of regulatory controls. The agents that function in this manner have pleiotropic effects. They are capable of eliciting significant changes in the cellular milieu of growth factors, growth factor receptors, signal transduction molecules, and other regulatory molecules. This mechanism may lead to direct effects on the PSA gene. Although PSA is often thought of as an androgen-regulated gene, it has been shown that PSA expression can be regulated by a variety of growth factors as well as by extracellular matrix proteins (Guo et al., 1994). Regulation of PSA may also occur post-transcriptionally. In this case, regulatory controls may exist for the degradation of PSA mRNA, translation of the mRNA into protein, post-translational modifications to PSA protein, and in the trafficking of the protein to be secreted. The agents that appear to act in this manner include gallium nitrate, CAI, troglitazone, and thalidomide. It is possible that a particular pharmacological agent might function in both an androgen-dependent and -independent manner. In this case, changes in the concentration of the agent, the presence or absence of interacting agents, and the general state of the cell might drive the regulation of PSA in one direction or the other. Alternatively, both mechanisms may function simultaneously leading to multiple levels of regulation.

These findings suggest that the efficacy of a particular pharmacological agent in the treatment of prostate cancer should be evaluated with caution. An agent should

not be assumed to have activity because it down-regulates PSA expression/secretion. Likewise, a drug should not be designated as inactive solely on the basis that it up-regulates PSA production. The effect of the agent on PSA expression should be assessed using a preclinical model system. There are caveats about the use of pre-clinical models to assess the effects of pharmacological agents on PSA regulation. Preclinical models are not predictive in all patients, and it is important to determine to what extent the results predicted correlate with clinical outcome. Recently, it has been shown that the results of *in vitro* experiments may be altered depending on the choice of preclinical model. For example, Davis and Sarkar showed that when LNCaP cells (which have a mutated AR) were exposed to genistein, PSA secretion was reduced. However, exposure of VCaP cells (which have a normal AR) to genistein had no effect on PSA secretion (Davis and Sarkar, 2000). These findings accentuate the necessity for the development of new model systems for the study of prostate cancer as well as the need to test hypotheses in multiple model systems. Pre-clinical models also isolate the effects of the agent to a particular cell type and culture condition. They do not take into account contributions from the surrounding cellular environment. These findings call for refinements in drug development for prostate cancer to involve careful preclinical work in both *in vitro* and *in vivo* model systems to document the possibility of regulation of PSA before attempting to interpret changes in PSA levels in clinical trials. In cases where PSA regulation is clearly altered independently of any antitumor effect, it should be noted in the design, conduct, and interpretation of clinical trials. Caution should be advised in the interpretation of PSA levels as an endpoint in clinical trials for agents in prostate cancer unless an exploration of changes in these PSA levels, independent of tumor activity, has been properly performed. Additionally, we need to develop new methods and models to assess the regulation of PSA expression/secretion *in vivo* as well as to prospectively validate the results obtained *in vitro* for clinical correlates.

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