Androgen Antagonist Activity by the Antioxidant Moiety of Vitamin E, 2,2,5,7,8-Pentamethyl-6-chromanol in Human Prostate Carcinoma Cells[1]

Todd A. Thompson and George Wilding[2]

University of Wisconsin Comprehensive Cancer Center [T. A. T., G. W.] and University of Wisconsin Department of Medicine [G. W.], University of Wisconsin-Madison, Madison, Wisconsin 53792

Abstract

Antioxidants, such as vitamin E, are being investigated for efficacy in prostate cancer prevention. In this study, we show that the antioxidant moiety of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol (PMCol), has antiandrogen activity in prostate carcinoma cells. In the presence of PMCol, the androgen-stimulated biphasic growth curve of LNCaP human prostate carcinoma cells was shifted to the right. The PMCol-induced growth shift was similar to that produced by treatment with the pure antiandrogen bicalutamide (*i.e.,* Casodex), indicative of androgen receptor (AR) antagonist activity. The concentration of PMCol used was below the concentration required to affect cell growth or viability in the absence of androgen. Using an AR binding competition assay, PMCol was found to be a potent antiandrogen in both LNCaP and LAPC4 cells, with an IC_{50} of approximately 10 μM against 1 nM R1881 (methyltrienolone; a stable, synthetic androgen). Prostate-specific antigen release from LNCaP cells produced by androgen exposure with either 0.05 or 1.0 nM R1881 was inhibited 100% and 80%, respectively, by 30 μM PMCol. Also, PMCol inhibited androgen-induced promoter activation in both LNCaP and LAPC4 cells. However, PMCol did not affect AR protein levels, suggesting that the inhibitory effects of PMCol on androgenic pathways were not due to decreased expression of the AR. Therefore, growth modulation by the antioxidant moiety of vitamin E in androgen-sensitive prostate carcinoma cells is due, at least in part, to its potent antiandrogenic activity.

Introduction

The activity of androgens is tissue specific and mediated through the AR.[3] The disruption of androgens and AR activity alters the regulation of androgen-sensitive tissues, such as the prostate gland (1). In the prostate, androgens have a central role in normal glandular development and function (2). However, androgens are also necessary for the development of prostate cancer. The role of androgens in prostate cancer development is emphasized by the observation that eunuchs and men that have a mutation in 5α-reductase type II, an enzyme that converts testosterone to the more potent dihydrotestosterone, do not develop prostate cancer (3). The incidence of prostate cancer has continued to rise for the last two decades, currently affecting over 200,000 men in the United States each year (4). Agents that permit the necessary actions of androgen for normal tissue function while reducing the role of androgens in the pathogenesis of androgen-sensitive tissues may serve as a useful means of reducing prostate cancer development. Recently, several agents have been reported to prevent prostate cancer development, such as selenium, lycopene, and vitamin E (5). Due to the biochemical nature of these agents, they are believed to act primarily through antioxidant-related pathways. However, the scope of their biological activity has not been extensively investigated.

Vitamin E is a family of naturally occurring dietary factors, which were originally identified as necessary for reproduction (6). α-Tocopherol, the most potent form of vitamin E, has two main components, a 16-carbon phytyl chain and a chromanol moiety with four methyl group substitutions (7). Biologically, α-tocopherol is thought to act primarily as an antioxidant, reducing oxidative damage to lipids. The chromanol moiety of α-tocopherol is responsible for its antioxidant activity, whereas the phytyl chain increases the lipophilicity of α-tocopherol and contributes to its tissue and subcellular distribution (8). Cell culture studies using α-tocopherol are difficult to perform due to its limited water solubility. However, the antioxidant chromanol moiety of α-tocopherol, PMCol, which does not possess a phytyl chain, is sufficiently water soluble to permit studies in cell culture.

Most human prostate carcinoma cell lines are androgen independent. The LNCaP human prostate carcinoma cell line is one of the few cell lines to show demonstrable responses to androgen exposure (9). Interestingly, LNCaP cells produce a biphasic growth response to androgen exposure, with growth stimulation occurring at lower doses and growth

Received 5/7/03; revised 6/2/03; accepted 6/18/03.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This research, under Award Numbers DAMD17-98-1-8505 and DAMD17-99-1-9531, was supported by the Department of Defense Prostate Cancer Research Program, which is managed by the United States Army Medical Research and Materiel Command. CaP CURE also provided funding for this project.
[2] To whom requests for reprints should be addressed, at University of Wisconsin-Madison, University of Wisconsin Comprehensive Cancer Center, K5-601 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792-6164. Phone: (608) 263-8600; Fax: (608) 263-8613; E-mail: gxw@medicine.wisc.edu.

[3] The abbreviations used are: AR, androgen receptor; CSS, charcoal-stripped serum; MMTV, mouse mammary tumor virus; PMC, 2,2,5,7,8-pentamethylchroman; PMCol, 2,2,5,7,8-pentamethyl-6-chromanol; PSA, prostate-specific antigen.

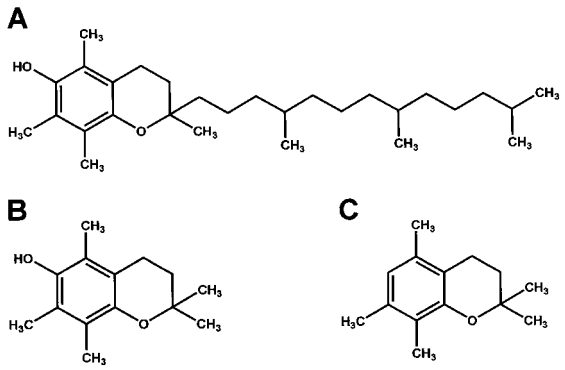

Fig. 1. Structure of vitamin E (*i.e.*, α-tocopherol) and related compounds. *A,* α-tocopherol. *B,* PMCol. *C,* PMC.

inhibition occurring in the absence of androgen or in the presence of high androgen levels (9, 10). In addition, a number of androgen-sensitive responses are induced in LNCaP cells. For example, LNCaP cells produce a dose-dependent increase in PSA expression on androgen exposure (11, 12). Also, androgen-sensitive promoters, such as the MMTV promoter, are activated by androgen in LNCaP cells (13). The exquisite sensitivity of LNCaP cells to androgenic stimulation may be due to a mutation in the ligand-binding domain of the AR (14). To date, the LNCaP prostate cell line has been the most extensively characterized prostate cell line for examining the effects of androgens. More recently, the LAPC4 cell line has been introduced as another androgen-sensitive human prostate carcinoma cell line that expresses a normal AR (15). However, the response of LAPC4 cells to androgens is not as pronounced as that observed in LNCaP cells. Collectively, the LNCaP and LAPC4 human prostate carcinoma cell lines provide valuable models for investigating androgen-regulated cellular pathways.

Studies on the actions of vitamin E and vitamin E analogues on prostate carcinoma cells have only recently begun. Previous studies have focused primarily on the inhibition of prostate cell growth by vitamin E treatment, which may occur through effects on cell cycle regulators (16–18). Apoptotic responses induced by vitamin E treatment have also been observed in LNCaP cells (19, 20). Interestingly, vitamin E-induced apoptotic responses were enhanced by coadministration of androgen (19). Zhang *et al.* (21) reported that vitamin E succinate reduces the levels of the AR in LNCaP cells, with resultant inhibition of androgen-mediated responses. However, the direct actions of vitamin E and related compounds on AR activity in prostate cells have not been extensively examined. In the current study, the AR antagonist activity and modulation of androgen-sensitive pathways by the vitamin E derivative PMCol were investigated in human prostate carcinoma cells.

Materials and Methods

Chemicals. PMCol and PMC were obtained from Aldrich (Milwaukee, WI). The chemical structures of α-tocopherol, PMCol, and PMC are shown in Fig. 1. Bicalutamide (Casodex) was kindly provided by AstraZeneca Pharmaceuticals (Wilmington, DE). R1881 (methyltrienolone) and ^{3}H-R1881 (87 Ci/mmol) were obtained from Perkin-Elmer/New England Nuclear Life Science Products (Boston, MA). All other chemicals used in these studies were acquired from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. LNCaP cells were acquired from American Type Culture Collection (Manassas, VA), and LAPC4 cells were kindly provided by Dr. Robert Reiter (University of California-Los Angeles) and maintained in DMEM containing 5% heat-inactivated FCS (Sigma) with streptomycin-penicillin antibiotics (designated DMEM/fetal bovine serum) in a 5% CO_2 incubator at 37°C. For experiments evaluating androgenic responses, cells were cultured in phenol red-free DMEM (Invitrogen, Carlsbad, CA) containing 4% charcoal-stripped FCS and 1% unstripped FCS (designated DMEM/CSS).

AR Binding Competition Assay. An AR binding competition assay was performed as described previously (22). LNCaP or LAPC4 prostate carcinoma cells were plated in 12-well tissue culture dishes (Costar, Corning, NY) at 3.0×10^5 cells/well in phenol-red free DMEM/CSS 3 days before analysis. For competitor analysis, DMEM/CSS was removed by aspiration and replaced with 1 ml of phenol-red free DMEM containing 1 nM ^{3}H-R1881, 1 μM triamcinolone acetonide, and competitor at the specified concentrations for 2 h at 37°C in a 5% CO_2 incubator. After incubation, competitor solution was aspirated, and cells were removed from the plate by trypsinization and placed in 12 × 75-mm polystyrene tubes. Cells were washed twice with 1 ml of phenol red-free DMEM and placed in 8.0 ml of ScintiVerse II Scintillation Cocktail (Fisher Scientific, Pittsburgh, PA) for determination of radioactivity (*i.e.*, dpm) using a Beckman LS 6000TA Liquid Scintillation System (Beckman Instruments Inc., Fullerton, CA).

Cell Growth and Viability Analyses. Five thousand LNCaP or LAPC4 cells were plated in each well of 96-well plates (Costar) in 100 μl of DMEM/CSS. Two to 3 days after plating, cells were treated by adding 100 μl of DMEM/CSS containing 2× the concentration of the specified treatment to each well. Four days after treatment, the relative cell number was estimated by determining the DNA concentration of each well using a Hoechst-based fluorescence DNA assay, as described previously (23). Growth analysis with DU145 cells was performed in a manner similar to that described for LNCaP and LAPC4 cells, except that DU145 cells were initially seeded at 500 cells/well. Cell viability was determined by trypan blue exclusion and quantified by light microscopic analysis using a hemacytometer.

Determination of Secreted PSA Levels. LNCaP cells were cultured in 96-well plates (Costar) at 5000 cells/well in DMEM/CSS 1 day before treatment. Forty-eight h after treatment, PSA levels in cell culture media were determined using the Tandem-MP PSA kit (Beckman Coulter, Inc.) according to the manufacturer's instructions. PSA levels were normalized to DNA levels as determined using a Hoechst-based fluorescence DNA assay (23).

Androgen-stimulated Promoter Reporter Assay Analysis. LNCaP and LAPC4 prostate carcinoma cell lines were cultured in 12-well cell culture plates (Costar) in DMEM/CSS

2–3 days before transfection. Androgen-induced trancriptional activation was determined using a reporter construct with a MMTV promoter that regulates the expression of luciferase (24). LNCaP and LAPC4 cells were transfected with the MMTV/luciferase plasmid using the Effectene Transfection Reagent (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. Twenty-four h after transfection, cells were treated with R1881 with or without test reagents at the specified concentrations. Cell extracts were acquired 24–48 h after treatment by removing medium, washing 1× with PBS, and obtaining extract with 200 μl of 1× Reporter Lysis Buffer (Promega, Madison, WI). Luciferase activity was determined as described previously (24).

Immunoblot Analysis of AR Protein Levels. LNCaP cells were plated at a density of 1×10^6 cells/100-mm cell culture plate in 10 ml of DMEM/fetal bovine serum and maintained in incubators at 37°C in 5% CO_2. After 5 days of treatment with vehicle, 30 μM PMC, 30 μM PMCol, or 1.0 μM bicalutamide, cells were washed in cold 1× PBS and lysed in a buffer containing 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/ml aprotinin in 1× PBS. Total protein (10 μg) from cell extracts was electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a GENIE wet transfer system (Idea Scientific, Minneapolis, MN). Membranes were blocked in Tris-buffered saline containing 5% nonfat dry milk and then incubated with mouse anti-AR monoclonal antibody (441; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse antiactin antibody (A5441; Sigma). Membranes were then incubated with a secondary horseradish peroxidase-conjugated antimouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed using Enhanced Chemiluminescence Plus reagent (Amersham Pharmacia Biotech). Autoradiograms were prepared by exposing the blots to BioMax Light X-ray film (Eastman Kodak Co., Rochester, NY) and developed using a CURIX 60 CP Processor (Agfa, Ridgefield Park, NJ).

Statistical Analysis. Significant differences in values between groups were assessed using a two-sided Student's *t* test. *P*s less than 0.05 were used to signify statistical significance.

Results

PMCol Inhibits Androgen Binding in Prostate Cancer Cells. AR competition was determined using ^{3}H-R1881 in the androgen-sensitive LNCaP cell line, which expresses a functional mutant AR (25), and the LAPC4 cell line, which express a normal human AR (15). Increasing concentrations of the AR antagonist bicalutamide were found to progressively inhibit R1881 binding (Fig. 2*A*), with an estimated IC_{50} of 0.7 μM in LNCaP cells. PMCol was found to be approximately 10-fold less potent at competing for ^{3}H-R1881 than bicalutamide in LNCaP cells, with an estimated IC_{50} of 7.2 μM (Fig. 2*A*). Repeated studies of PMCol competition for ^{3}H-R1881 binding gave IC_{50} values ranging from 5 to 15 μM (data not shown). In contrast, PMC, in which the 6-hydroxyl of PMCol is absent, had less antiandrogenic activity than PMCol (Fig. 2*A*) and significantly reduced cell viability at a concentration of 100 μM within 2 h of treatment (data not shown). Based on the R1881 competition results in LNCaP cells (Fig. 2*A*), a dose of 30 μM PMC and PMCol was used in most of these studies, allowing an effective comparison of the antiandrogenic activity between PMC and PMCol. In LAPC4 cells, treatment with 30 μM PMCol produced a 75% decrease in ^{3}H-R1881 binding, and treatment with 1 μM bicalutamide produced a 62% decrease in ^{3}H-R1881 binding (Fig. 2*B*).

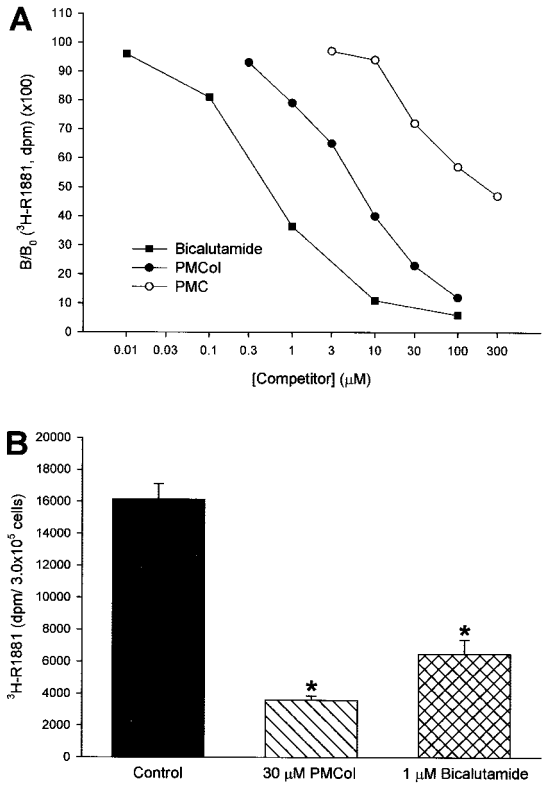

Fig. 2. PMCol competition analysis of R1881 binding in human prostate carcinoma cells. *A,* dose response for the competition of PMCol, PMC, and bicalutamide for AR binding to ^{3}H-R1881 was determined in LNCaP cells. *B,* competition for ^{3}H-R1881 binding in LAPC4 cells was determined for 30 μM PMCol and 1 μM bicalutamide. ∗, $P < 0.05$; $n = 4$.

Modulation of Prostate Carcinoma Cell Growth and Viability by PMCol. Changes in growth of the androgen-independent DU145 prostate carcinoma cell line and the androgen-sensitive LNCaP and LAPC4 prostate cell lines were assessed at concentrations of PMCol ranging from 10 to 100 μM (Fig. 3*A*). Concentrations of 50, 60, and 80 μM PMCol were required to significantly reduce cell growth in LNCaP, LAPC4, and DU145 cells, respectively (Fig. 3*A*). LNCaP cells produce a biphasic growth response to androgen exposure (9). Modulation of LNCaP cell growth by PMCol treatment was examined over 4 days. PMCol had no growth-modulatory activity in vehicle control-treated LNCaP cells grown in androgen-deficient media (*i.e.*, PMCol did not have AR agonist activity) at concentrations ranging from 10 to 30 μM PMCol (Fig. 3*B*). However, LNCaP cell growth was decreased at concentrations equal to or higher than 40 μM

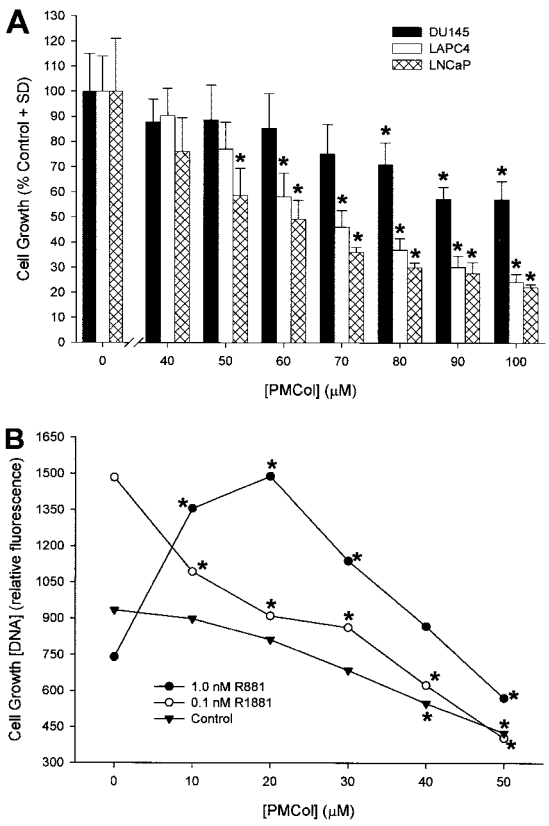

Fig. 3. Growth modulation of human prostate carcinoma cells by PMCol. *A,* dose response of DU145, LAPC4, and LNCaP cells grown in medium containing 5% serum measured 4 days after PMCol treatment. Treatment with 50 μM PMCol significantly reduced LNCaP prostate cell growth, whereas a concentration of 80 μM PMCol was required to significantly decrease growth in the androgen-independent DU145 prostate cell line (*, $P < 0.05$). *B,* the PMCol dose response of LNCaP cell growth was determined in cells exposed to androgen-deficient conditions (*i.e.*, using medium containing reduced androgen levels) with or without the addition of a growth-stimulatory dose of 0.05 nM R1881 or a growth-inhibitory dose of 1.0 nM R1881. *, significantly different than 0 μM PMCol-treated cells, $P < 0.05$; $n = 6$.

PMCol (Fig. 3*B*), and PMCol concentrations of ≥100 μM produced significant cell death at 48 and 96 h (Table 1). Stimulation of LNCaP growth by exposure to 0.1 nM R1881 was significantly inhibited by treatment with concentrations of ≥10 μM PMCol (Fig. 3*B*). However, a significant stimulation in LNCaP cell growth was observed in the presence of a normally growth-inhibitory concentration of 1.0 nM R1881 with treatment of 10–30 μM PMCol (Fig. 3*B*). The R1881-stimulated growth curve of LNCaP cells was shifted to the right in the presence of 30 μM PMCol, similar to that produced by treatment with 1 μM bicalutamide (Fig. 4). A more modest, but significant, shift to the right in the androgen-induced LNCaP growth curve was observed by treatment with 30 μM PMC (Fig. 4).

Inhibition of PSA Secretion by PMCol in LNCaP Cells. PSA secretion by LNCaP cells is stimulated by androgen exposure in a dose-dependent manner (12). The R1881-stimulated production of PSA from LNCaP cells was measured after PMCol treatment for 48 h. PSA release from LNCaP cells was not affected by treatment with 30 μM PMCol alone (Fig. 5). However, PSA levels were increased 3.1-fold after exposure to a growth-stimulatory dose of 0.05 nM R1881, which was completely inhibited by treatment with 30 μM PMCol (Fig. 5). Exposure of LNCaP cells to 1.0 nM R1881 produced a 12-fold increase in PSA levels by 48 h, which was decreased 20%, 81%, and 43% by treatment with 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide, respectively (Fig. 5).

Inhibition of Androgen-stimulated Transcriptional Activation by PMCol. Studies on androgen-regulated transcriptional activation were performed in LNCaP and LAPC4 cells transiently transfected with a reporter vector that uses the androgen-sensitive MMTV long terminal repeat to drive expression of a luciferase reporter gene. In LNCaP cells, PMCol treatment alone had no effect on MMTV promoter activity, whereas luciferase expression was increased 54-fold after exposure to 1.0 nM R1881 for 24 h (Fig. 6*A*). Luciferase expression induced by exposure to 1.0 nM R1881 in LNCaP cells for 24 h was decreased 50% and 70% by treatment with 25 and 50 μM PMCol, respectively (Fig. 6*A*). Similarly, LAPC4 cells exposed to 1.0 nM R1881 produced a 20-fold increase in MMTV long terminal repeat-driven luciferase expression that was decreased 60% by treatment with 30 μM PMCol after 24 h (Fig. 6*B*). In both LNCaP and LAPC4 cells, treatment with 1 μM bicalutamide decreased 1.0 nM R1881-stimulated luciferase expression approximately 50% (Fig. 6, *A* and *B*).

AR Protein Levels in PMCol-exposed LNCaP Cells. Previous studies in LNCaP cells have reported that AR levels are decreased after treatment with vitamin E analogues, which may account for the reduced sensitivity of these cells to androgen exposure (21). However, in the current study, treatment with 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide for 5 days did not result in altered AR protein levels in LNCaP cells (Fig. 7).

Discussion

In the current study, we examine the effects of an agent traditionally considered as an antioxidant on prostate carcinoma cells. Epidemiological studies provide intriguing evidence that antioxidant dietary factors such as β-lycopene and vitamin E may help prevent prostate cancer development (5). Although these agents have been classified as antioxidants, the mechanism by which they may contribute to prostate cancer prevention has not been firmly established. Androgens are known to have an essential role in prostate cancer development (3). Modulation of androgen activity may provide a means of prostate cancer prevention (26). Here, we report the antioxidant moiety of vitamin E, PMCol, to be a potent antiandrogen in androgen-sensitive human prostate carcinoma cells.

The LNCaP human prostate carcinoma cell line is one of the few prostate cell lines that show demonstrable physiological changes resulting from androgen exposure, such as growth modulation (9). Therefore, the LNCaP cell line has proven valuable in identifying agents that alter androgen-stimulated cell growth. In the current study, PMCol shifted the androgen-mediated growth curve in LNCaP cells such

Table 1 Time- and dose-dependent changes in LNCaP cell viability after PMCol treatment

Time (h)	% Cell viability[a] (SD)					
	PMCol (μM)					
	0	25	50	75	100	250
48	92.3 (4.7)	90.0 (2.8)	88.0 (3.4)	80.0 (12.5)	71.0 (8.7)[b]	11.0 (8.6)[b]
96	88.0 (2.5)	87.0 (4.8)	85.0 (4.6)	87.0 (4.0)	21.0 (3.3)[b]	2.0 (1.8)[b]

[a] Determined by trypan blue exclusion analysis and quantified using a hemacytometer.
[b] Significantly different compared to 0 μM PMCol ($P < 0.05$; $n = 4$).

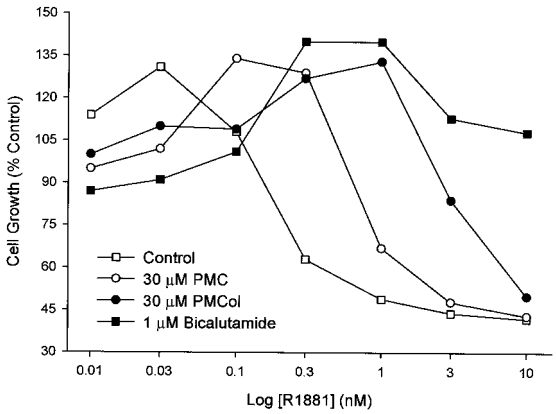

Fig. 4. Shifts in the R1881-stimulated biphasic LNCaP growth response were determined after treatment with 30 μM PMCol, 30 μM PMC, or 1 μM bicalutamide for 4 days. The inhibition of growth response is readily apparent at 0.3 nM R1881 exposure, where LNCaP growth from PMCol, PMC, and bicalutamide treatment was equivalent to the growth response in control cells produced by exposure to only 0.03 nM R1881.

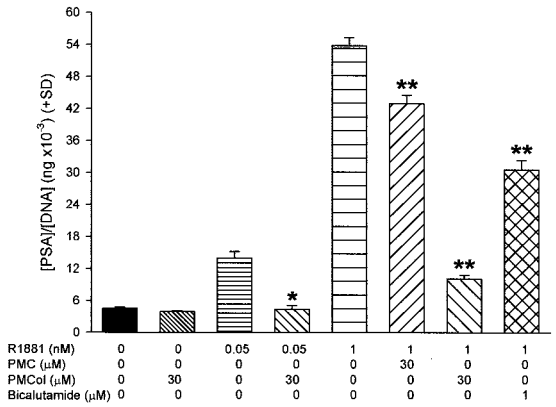

Fig. 5. Analysis of PMCol effects on androgen-induced PSA secretion from LNCaP cells. PSA secretion was determined 48 h after exposure to a growth-stimulatory dose of 0.05 nM R1881 or a growth-inhibitory dose of 1.0 nM R1881 in the presence of 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide. *, $P < 0.05$ compared with 0.05 nM R1881-treated cells; **, $P < 0.05$ compared with 1.0 nM R1881-treated cells; $n = 3$.

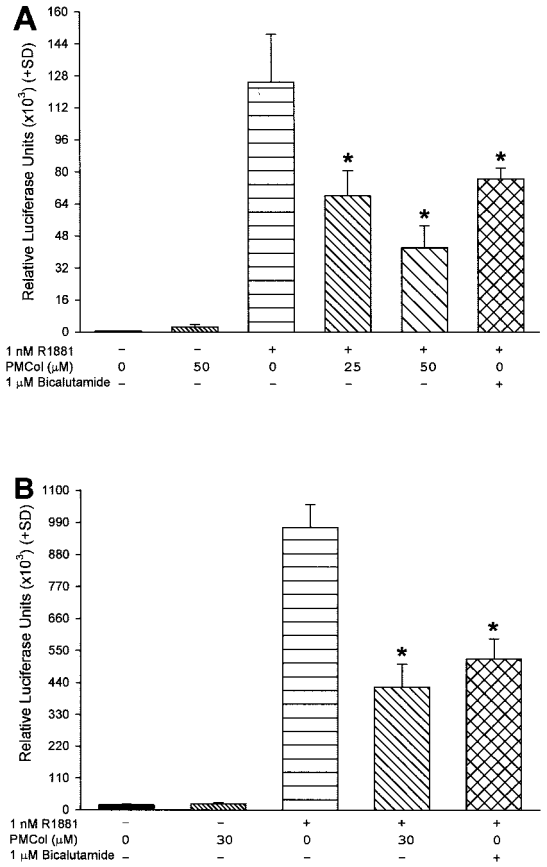

Fig. 6. Androgen-induced MMTV promoter activity in LNCaP (*A*) and LAPC4 (*B*) cells after PMCol treatment. *A,* the effects of 25 μM PMCol, 50 μM PMCol, and 1 μM bicalutamide treatment for 24 h on MMTV promoter activity induced by R1881 were assessed in LNCaP cells. *B,* LAPC4 cells exposed to 30 μM PMCol effectively inhibited androgen-induced MMTV promoter activity. *, $P < 0.05$; $n = 4$.

that higher androgen concentrations were necessary to produce the biphasic growth response typically observed in LNCaP cells. The LNCaP growth shift with PMCol treatment was sufficient to produce growth stimulation in the presence of 1.0 nM R1881, a concentration of R1881 that typically inhibits LNCaP proliferation (10). The shift in LNCaP growth pattern observed with PMCol treatment was similar to that observed in LNCaP cells after treatment with the pure antiandrogen bicalutamide. Also, the IC_{50} of PMCol observed in an androgen competition analysis for R1881 binding in LNCaP cells is in agreement with the dose-response shift in androgen-mediated growth of LNCaP cells after PMCol treatment. Together, these results suggest that the shift observed in the androgen-modulated growth of LNCaP cells was due to the antiandrogenic activity of PMCol.

Although LNCaP cells have proven to be useful in evaluating androgen-responsive pathways, the use of LNCaP cells

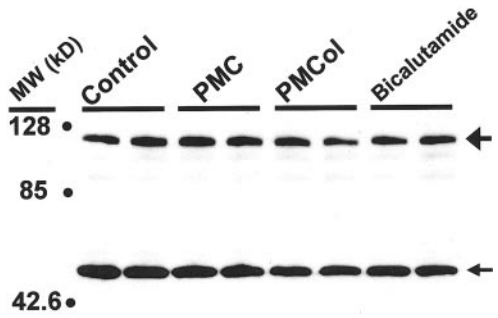

Fig. 7. Immunoblot analysis of AR protein levels. AR protein levels were not significantly altered in LNCaP cells exposed to 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide for 5 days compared with AR levels in vehicle control-exposed cells. LNCaP cells were grown in medium containing 5% serum to provide endogenous serum androgens, thus allowing antiandrogenic modulation of AR protein levels. The *large arrow* points to AR protein bands, and the *small arrow* points to β-actin protein bands.

to assess antiandrogenic activity can be inaccurate because LNCaP cells harbor a mutant AR (25). The AR in LNCaP cells, although functional, has been reported to have altered ligand binding affinity (14) and is stimulated by some agents that are antagonists for the wild-type AR (22). Therefore, in this study, competition for AR binding by PMCol was also assessed in the LAPC4 human prostate carcinoma cell line, which expresses a wild-type AR (15). PMCol competition for R1881 binding was found to be similar for LNCaP and LAPC4 cells. In addition, the pure antiandrogen bicalutamide was found to have equivalent AR competition activity in LNCaP and LAPC4 cells. Therefore, the pure antiandrogen bicalutamide and PMCol were found to possess comparable AR antagonist activity in LNCaP cells, which express a functional mutant AR, and LAPC4 cells, which express a normal AR.

The AR functions primarily as a transcription factor that is activated by androgen binding (1). In these studies, the androgen-responsive MMTV promoter was used to assess modulation of androgen-stimulated transcriptional activity. Upon androgen exposure (*i.e.*, R1881), MMTV promoter activity was stimulated in both LNCaP and LAPC4 cells. Also, in both cell lines, R1881 stimulation of MMTV activity was significantly inhibited by PMCol treatment. PMCol treatment alone did not stimulate MMTV promoter activity (*i.e.*, PMCol was not found to have AR agonist or partial agonist activity). The effects of androgen exposure on transcriptional activation were further observed by the inhibition of androgen-stimulated PSA release after treatment with PMCol in LNCaP cells. Previously, vitamin E succinate was reported to inhibit the effects of androgen on LNCaP cells through down-regulation of AR levels (21). Other agents, such as curcumin, have been shown to decrease AR expression in LNCaP cells (27). In the current study, treatment of LNCaP cells with 30 μM PMCol for 5 days did not affect AR protein levels. Therefore, PMCol was found to be a potent inhibitor of transcriptional activation of androgen-responsive promoters, likely through directly blocking of AR activation by androgen.

Understanding how structure-activity relationships of the chromanol ring of PMCol contribute to antiandrogenic activity may prove useful in developing potent chromanol ring-based nonsteroidal antiandrogenic agents. In the current study, PMC, which lacks the phenolic hydroxyl group present on PMCol, was less potent than PMCol at inhibiting androgenic responses. Therefore, the phenolic hydroxyl group of the chromanol ring contributes significantly to the antiandrogenic activity of PMCol. Other forms of vitamin E, such as β-, γ-, and δ-tocopherol differ from α-tocopherol by the number and location of methyl group substitutions on the chromanol ring (7). We can only speculate that the antioxidant moieties of other forms of vitamin E also possess antiandrogenic activity with potencies that vary depending on the specific methyl group substitutions present on the chromanol ring.

A variety of dietary agents have been identified that have antiandrogenic activity in prostate carcinoma cells. However, the mechanism of antiandrogenic activity observed by dietary antiandrogens may vary. For example, curcumin, a component of turmeric, was reported to down-regulate AR protein levels in LNCaP cells, which effectively attenuates androgenic responses (27). In contrast, indole-3-carbinol, a component of cruciferous vegetables, when converted to diindolylmethane was reported to act as a potent inhibitor of androgen binding in LNCaP cells but does not affect AR protein levels (28). Zhang *et al.* (21) have reported that vitamin E succinate is inhibitory to androgenic responses in LNCaP cells through down-regulation of AR protein levels, similar to the action of curcumin. By contrast, in the current study, we found that the antioxidant moiety of vitamin E, PMCol, effectively blocks androgen binding to the AR without affecting AR protein levels, similar to the effects observed with indole-3-carbinol derivatives (28). Therefore, dietary antiandrogens may serve as an effective means of modulating androgenic pathways through a variety of mechanisms affecting AR activity.

It is unclear how accurately the biological activity of α-tocopherol is modeled by the PMCol antioxidant moiety alone. PMCol has largely been investigated for its antioxidant activity associated with being the antioxidant moiety of vitamin E. For example, the antioxidant potency of PMCol was shown to be similar to that of α-tocopherol *in vitro* (29). In general, α-tocopherol plasma levels range between 5 and 30 μM (30), well within the range of antiandrogenic activity observed by PMCol in the current study. Due to the high lipophilicity of vitamin E, it is difficult to assess its antiandrogenic activity by cell culture analysis. However, we can speculate that due to the presence of the highly lipophilic phytyl chain, the subcellular distribution of vitamin E would limit its direct interaction with the AR, which resides in more aqueous subcellular compartments such as the cytoplasm and nucleus. Vitamin E can be metabolized to derivatives with greater water solubility, such as α-carboxyethylhydroxychroman (7, 31), which are structurally similar to PMCol and may have greater water solubility and a distinct cellular bioavailability compared with vitamin E. Thus, we hypothesize that metabolites of vitamin E may contact the AR *in vivo* and have antiandrogenic activity, analogous to that produced by PMCol in human prostate carcinoma cells.

In summary, the antioxidant moiety of α-tocopherol, PMCol, was found to inhibit androgen activity, likely through

competition for androgen binding to the AR, with resultant inhibition of androgen-sensitive biological pathways. PMCol was not found to possess androgen agonist or partial agonist activity and hence functions as a pure antagonist of androgen activity in the LNCaP and LAPC4 prostate carcinoma cell lines. Based on the results of the current study, PMCol may serve as a useful agent for modulating androgen activity *in vivo*. Importantly, the antiandrogenic activity of PMCol poses the possibility that the prostate cancer-preventive activity of vitamin E may be due, in part, to antiandrogenic effects of vitamin E or metabolites of vitamin E in the prostate. Currently, over 30,000 men die from prostate cancer each year in the United States (4). The prevention of prostate cancer through the action of dietary antiandrogens, such as vitamin E or its derivatives, may offer one means of reducing the devastation produced by this disease.

Acknowledgments

We thank Robert Kolb for excellent technical assistance.

References

1. Lamb, D. J., Weigel, N. L., and Marcelli, M. Androgen receptors and their biology. Vitam. Horm., *62:* 199–229, 2001.

2. Wilding, G. The importance of steroid hormones in prostate cancer. Cancer Surv., *14:* 113–130, 1992.

3. Wilding, G. Endocrine control of prostate cancer. Cancer Surv., *23:* 43–62, 1995.

4. Jemal, A., Murray, T., Samuels, A., Ghafoor, A., Ward, E., and Thun, M. J. Cancer statistics, 2003. CA Cancer J. Clin., *53:* 5–26, 2003.

5. Gronberg, H. Prostate cancer epidemiology. Lancet, *361:* 859–864, 2003.

6. Evans, H. M., and Bishop, K. S. On the existence of a hitherto unrecognized dietary factor essential for reproduction. Science (Wash. DC), *56:* 650–651, 1922.

7. Brigelius-Flohe, R., and Traber, M. G. Vitamin E: function and metabolism. FASEB J., *13:* 1145–1155, 1999.

8. Burton, G. W., and Traber, M. G. Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annu. Rev. Nutr., *10:* 357–382, 1990.

9. Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A., and Murphy, G. LNCaP model of human prostatic carcinoma. Cancer Res., *43:* 1809–1818, 1983.

10. Ripple, M. O., Henry, W. F., Rago, R. P., and Wilding, G. Prooxidant-antioxidant shift induced by androgen treatment of human prostate carcinoma cells. J. Natl. Cancer Inst. (Bethesda), *89:* 40–48, 1997.

11. Riegman, P. H., Vlietstra, R., J., van der Korput, J. A., Brinkmann, A. O., and Trapman, J. The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol. Endocrinol., *5:* 1921–1930, 1991.

12. Young, C. Y., Montgomery, B. T., Andrews, P. E., Qui, S. D., Bilhartz, D. L., and Tindall, D. J. Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. Cancer Res., *51:* 3748–3752, 1991.

13. Warriar, N., Page, N., Koutsilieris, M., and Govindan, M. V. Antiandrogens inhibit human androgen receptor-dependent gene transcription activation in the human prostate cancer cells LNCaP. Prostate, *24:* 176–186, 1994.

14. Veldscholte, J., Berrevoets, C. A., and Mulder, E. Studies on the human prostatic cancer cell line LNCaP. J. Steroid Biochem. Mol. Biol., *49:* 341–346, 1994.

15. Klein, K. A., Reiter, R. E., Redula, J., Moradi, H., Zhu, X. L., Brothman, A. R., Lamb, D. J., Marcelli, M., Belldegrun, A., Witte, O. N., and Sawyers, C. L. Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. Nat. Med., *3:* 402–408, 1997.

16. Israel, K., Sanders, B. G., and Kline, K. RRR-α-tocopherol succinate inhibits the proliferation of human prostatic tumor cells with defective cell cycle/differentiation pathways. Nutr. Cancer, *24:* 161–169, 1995.

17. Venkateswaran, V., Fleshner, N. E., and Klotz, L. H. Modulation of cell proliferation and cell cycle regulators by vitamin E in human prostate carcinoma cell lines. J. Urol., *168:* 1578–1582, 2002.

18. Ni, J., Chen, M., Zhang, Y., Li, R., Huang, J., and Yeh, S. Vitamin E succinate inhibits human prostate cancer cell growth via modulating cell cycle regulatory machinery. Biochem. Biophys. Res. Commun., *300:* 357–363, 2003.

19. Gunawardena, K., Murray, D. K., and Meikle, A. W. Vitamin E and other antioxidants inhibit human prostate cancer cells through apoptosis. Prostate, *44:* 287–295, 2000.

20. Israel, K., Yu, W., Sanders, B. G., and Kline, K. Vitamin E succinate induces apoptosis in human prostate cancer cells: role for Fas in vitamin E succinate-triggered apoptosis. Nutr. Cancer, *36:* 90–100, 2000.

21. Zhang, Y., Ni, J., Messing, E. M., Chang, E., Yang, C. R., and Yeh, S. Vitamin E succinate inhibits the function of androgen receptor and the expression of prostate-specific antigen in prostate cancer cells. Proc. Natl. Acad. Sci. USA, *99:* 7408–7413, 2002.

22. Wilding, G., Chen, M., and Gelmann, E. P. Aberrant response *in vitro* of hormone-responsive prostate cells to antiandrogens. Prostate, *14:* 103–115, 1989.

23. Rago, R., Mitchen, J., and Wilding, G. DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water. Anal. Biochem., *191:* 31–34, 1990.

24. Thompson, T. A., Gould, M. N., Burkholder, J. K., and Yang, N-S. Transient promoter activity in primary rat mammary epithelial cells evaluated using particle bombardment gene transfer. In Vitro Cell. Dev. Biol., *29A:* 165–170, 1992.

25. Veldscholte, J., Ris-Stalpers, C., Kuiper, G. G., Jenster, G., Berrevoets, C., Claassen, E., van Rooij, H. C., Trapman, J., Brinkmann, A. O., and Mulder, E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem. Biophys. Res. Commun., *173:* 534–540, 1990.

26. Lieberman, R. Androgen deprivation therapy for prostate cancer chemoprevention: current status and future directions for agent development. Urology, *58:* 83–90, 2001.

27. Nakamura, K., Yasunaga, Y., Segawa, T., Ko, D., Moul, J. W., Srivastava, S., and Rhim, J. S. Curcumin down-regulates AR gene expression and activation in prostate cancer cells. Int. J. Oncol., *21:* 825–830, 2002.

28. Le, H. T., Schaldach, C. M., Firestone, G. L., and Bjeldanes, L. F. Plant-derived 3,3′-diindolylmethane is a strong androgen antagonist in human prostate cancer cells. J. Biol. Chem., *278:* 21136–21145, 2003.

29. Burton, G. W., Cheeseman, K. H., Doba, T., Ingold, K. U., and Slater, T. F. Vitamin E as an antioxidant *in vitro* and *in vivo. In:* Biology of Vitamin E: Ciba Foundation Symposium 101, pp. 4–18. London: Pitman, 1983.

30. Dimitrov, N. V., Meyer, C., Gilliland, D., Ruppenthal, M., Chenoweth, W., and Malone, W. Plasma tocopherol concentrations in response to supplemental vitamin E. Am. J. Clin. Nutr., *53:* 723–729, 1991.

31. Traber, M. G., Elsner, A., and Brigelius-Flohe, R. Synthetic as compared with natural vitamin E is preferentially excreted as αCEHC in human urine: studies using deuterated α-tocopheryl acetates. FEBS Lett., *437:* 145–148, 1998.

Molecular Cancer Therapeutics

Androgen Antagonist Activity by the Antioxidant Moiety of Vitamin E, 2,2,5,7,8-Pentamethyl-6-chromanol in Human Prostate Carcinoma Cells[1]

Todd A. Thompson and George Wilding

Mol Cancer Ther 2003;2:797-803.