Novel Oligomeric Proanthocyanidin Derivatives Interact with Membrane Androgen Sites and Induce Regression of Hormone-Independent Prostate Cancer[S]

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ABSTRACT

Prostate cancer is the most common malignancy among men in Western societies, and current therapeutic approaches are evolving to manage growth, recurrence, and mortality neoplasia. Membrane androgen receptors (mARs) have been characterized in human prostate cancer, being preferentially expressed in tumor rather than benign gland areas. Furthermore, mAR agonists (protein-conjugated testosterone) decrease in vitro prostate cancer cell growth and induce apoptosis, whereas in vivo they regress growth of tumor xenografts alone or in combination with taxane drugs. In this respect, targeting mARs might be a novel therapeutic approach in prostate cancer. In our search for new small-molecule ligands of mAR, we report that flavanol dimers B1-B4 (oligomeric procyanidins) decrease in vitro growth of the androgen-sensitive (LnCaP) and androgen-resistant (DU145) human prostate cancer cell lines in the following order: B3 = B4 > B2 ≫ B1 (LnCaP) and B2 ≫ B3 = B4 ≫ B1 (DU145). Some of these analogs were previously shown to trigger signaling cascades similar to testosterone-bovine serum albumin (BSA) conjugate. Galloylation does not confer an additional advantage; however, oleylation increases the dimers' antiproliferative potency by a factor of 100. In addition, we report that B2, oleylated or not, displaces testosterone from mARs with an IC_{50} value at the nanomolar range and induces DU145 tumor xenograft regression by 50% (testosterone-BSA 40%). In this respect, oleylated B2 is a potent small-molecule agonist of mAR and could be a novel therapeutic agent for advanced prostate cancer, especially when taking into account the absence of androgenic actions and (liver) toxicity.

Introduction

Steroid effects are classically mediated through intracellular receptor proteins belonging to the nuclear receptor superfamily. Upon steroid binding, they dimerize, translocate to the nucleus, and act as ligand-activated transcription factors, modulating steroid-dependent genes (Kumar and Tindall, 1998). In recent years, however, an alternative mode of steroid action has been revealed that integrates rapid actions, initiated at the membrane level and leading to a multitude of cellular modifications, such as rapid ion movement through the plasma membrane, secretion modification, and initiation of signaling cascades (Hammes and Levin, 2007). The latter ultimately lead to transcriptional activation distinct from the one initiated by nuclear receptors (Notas et al., 2010). Rapid plasma membrane-related actions, although described as early as 1942 (Seyle, 1942), became a field of intense research only in the last decade. The nature of membrane steroid binding sites has not been unanimously accepted. Nonmutually exclusive possibilities include: 1) membrane anchoring of intracellular receptors through post-translational modifica-

ABBREVIATIONS: OPC, oligomeric proanthocyanidin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase; ER, estrogen receptor.

tions, acting independently or in association with growth factor receptors (Marino and Ascenzi, 2006), 2) truncated or alternative spliced steroid receptors (Wang et al., 2006), and 3) novel receptor proteins (Zhu et al., 2003). Indeed, a number of receptors have been reported, belonging mainly to the G protein-coupled receptor family, to mediate some of the membrane-initiated steroid effects. However, there is extensive discussion about whether these receptors are true steroid receptors or coreceptor proteins (Levin, 2009).

Even if the identification of androgen membrane receptors remains a field of extended exploration (Kampa et al., 2008), the repertoire of their membrane effects is widely accepted to lead to actin cytoskeleton modifications and specific genes' transcription independently from nuclear androgen receptor action (Notas et al., 2010). Membrane androgen binding sites have been reported in a number of normal or malignant tissues and lesions. In particular, androgen sites have been detected in T lymphocytes (Benten et al., 1999), spermocytes and sperm (Walker, 2003), breast cancer (Pelekanou et al., 2007), prostate cancer (Dambaki et al., 2005), and colon cancer (Gu et al., 2009). In both breast and prostate, membrane-acting androgen have been reported to induce tumor regression alone (Hatzoglou et al., 2005) or in combination with cytoskeletal acting drugs (Kampa et al., 2006), suggesting a potential therapeutic role of membrane androgen agonists in breast and prostate cancer. However, until recently, no specific agonists (except for large-molecule-conjugated androgen) have been described.

Polyphenol-rich foods and beverages have been implicated in the prevention of a number of chronic conditions, including cardiovascular diseases and cancer. Nevertheless, although a number of epidemiological and intervention studies demonstrate this beneficial effect, experimental data dealing with their mode of action are divergent. Indeed, polyphenols are considered to enter the cell (after a possible biotransformation) and modify a number of cellular responses, including signaling molecules, enzymes, and/or transcription factors, leading ultimately to modification of the cell fate, toward survival or apoptosis (see Kampa et al., 2002 for a review). It is noteworthy that a study also suggests a potential membrane-initiated action of catechin analogs (Bastianetto et al., 2009), interacting with protein kinase C isoforms, a finding compatible with our previous results on signaling cascades initiated by membrane-acting testosterone conjugates (Papakonstanti et al., 2003). In addition, we have previously reported that the flavanols catechin and epicatechin, and their dimers B5 and especially B2, are in vitro agonists of membrane androgen sites, activating focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K), modifying further actin polymerization and leading mammary adenocarcinoma cells to apoptosis (Nifli et al., 2005). In the present work, we initially assayed natural and modified proanthocyanidin derivatives B1-B4 on prostate cell lines. The best-performing molecules have been further investigated for binding affinity on membrane androgen sites and regressive activity in prostate cancer xenografts.

Materials and Methods

Cell Lines and Culture Conditions. LNCaP and DU-145 cells (DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK) and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. Testosterone-3-(O-carboxymethyl)-oxime-BSA (10 molecules of testosterone per molecule of BSA) was purchased from Sigma Hellas (Athens, Greece) and used dissolved in PBS buffer. Before each experiment, a new solution of BSA conjugate was prepared and subjected to treatment with dextran (0.05 mg/ml) and charcoal (50 mg/ml) for 30 min to remove any potential contamination with free testosterone (Hatzoglou et al., 2005). We assayed routinely culture media for the presence of free testosterone with a specific radioimmunoassay method with negative results. Cell growth was assayed by the tetrazolium salt assay.

Proanthocyanidin Isolation and Synthesis of Derivatives. Proanthocyanidins were all obtained from ethyl acetate grape seed extracts (*Vitis vinifera*, Vitaceae) on which centrifugal partition chromatography in hexane-ethyl acetate-ethanol-water (1:8:2:7; v/v/v/v) was applied as described previously (Delaunay et al., 2002). This quantitative process allowed us to separate seven blocks in the ascending mode. Isolation of every proanthocyanidin was then realized by preparative reverse C18 high-performance liquid chromatography (HPLC) of each block, using a 0 to 100% methanol-water gradient. Eleven proanthocyanidins were thus isolated as pure compounds and fully characterized. Their identification was ascertained by two complementary methods: 1) comparing two-dimensional long-range NMR spectra (gradient accelerated spectroscopy-heteronuclear multiple bond correlation) (Bax and Summers, 1986) of peracetylated proanthocyanidins with those of references (Balas et al., 1995), to establish their gross structure and 2) comparing chromatographic behavior of the adducts formed from each one upon acid hydrolysis in the presence of excess phloroglucinol (Kennedy and Jones, 2001) with those issued from reference samples, after coinjection on an analytical HPLC system, coupled to array detection online with an electrospray ionization mass spectrometer, to confirm the nature (catechin or épicatéchine series) of each moiety and ascertain the type of the interflavanolic linkage. HPLC conditions for analysis of proanthocyanidins (method 1) were: Column Synergi 4 hydro-RP 80A (250 × 2.0 mm) from Phenomenex (Torrance, CA); water-0.0025% trifluoroacetic acid (v/v, solvent A), methanol-0.0025% trifluoroacetic acid (v/v, solvent B); gradient: initial 85% A, from 15 to 50% B in 30 min, from 50 to 100% B in 3 min; detection at 280 nm, flow rate 0.2 ml/min. HPLC conditions for phloroglucinolysis analysis (method 2) were: Column Atlantis dC18 (4.6 × 250 mm) from Waters (Milford, MA); 2% aqueous formic acid (v/v, solvent A), acetonitrile/water/formic acid (80:18:2 v/v/v, solvent B); initial 100% A and during 8 min, from 0 to 20% B in 32 min, from 20 to 95% B in 5 min; detection at 280 nm, flow rate: 1 ml/min; oven temperature 30°C. Electrospray ionization mass spectrometry was done with a Thermo Finnigan LCQ Advantage detector from Thermo Fisher Scientific (Waltham, MA), monitored by Xcalibur 2.1 software (Thermo Fisher Scientific).

Eleven proanthocyanidins were isolated and identified, with HPLC method 1 as procyanidin B3 (R_t = 11.5 min), procyanidin B1 (R_t = 12.0 min), procyanidin B4 (R_t = 14.6 min), catechin (R_t = 15.5 min), procyanidin B2 (R_t = 17.1 min), procyanidin B1 3F-O-gallate (R_t = 18.3 min), procyanidin B2 3F-O-gallate (R_t = 19.4 min), epicatechin (R_t = 21.2 min), trimer 3-O-gallate (R_t = 22.3 min), epicatechin-3-O-gallate (R_t = 25.5 min), and procyanidin B2 3C, 3F-di-O-gallate (R_t = 33.0 min). These proanthocyanidins had already been isolated and identified in grapes (da Silva et al., 1991).

Preparation of Oleylated B2 and B3 Derivative. To a solution of dimer B2 or B3 (2 g; 3.5 mmol) in 300 ml of $CHCl_3$ was added 766 μl of triethylamine (556 mg, 5.49 mmol, 1.57 eq.), and the resulting mixture was stirred at room temperature. Oleyl chloride (1.653 g; 5.49 mmol, 1.57 eq.), dissolved in 200 ml of $CHCl_3$ was added dropwise over a 2-h period. The $CHCl_3$ used was checked to be free of any trace of ethanol (stabilized by amylene). The mixture was stirred at room temperature under nitrogen for an additional 6 h. Sodium bicarbonate aqueous solution was then added until the pH was made alkaline. A saturated solution of NH_4Cl was added before the extraction by $CHCl_3$. The organic layer was washed with water, dried over

sodium sulfate, filtered, and evaporated to dryness under reduced pressure. The crude extract was submitted to a flash chromatography on a column of silica, and the major product was collected in 52% yield. It was shown to be the dioleylated derivative of B2 or B3: the electrospray ionization mass spectrometer in the negative ionization mode exhibited a signal of pseudomolecular ion $[M-H]^-$ in favor of a dioleyl ester derivative with a molecular mass $M = 1106$. The IR spectrum showed the characteristic band for such ester groups at 1769 cm^{-1} (-O–C = O aromatic esters). Although NMR clearly confirmed the presence of two oleyl residues, it was not possible to unambiguously determine which phenolic groups on rings B and/or E were esterified.

Binding Experiments. Cultured cells were washed with phosphate-buffered saline (PBS), removed by scrapping, and centrifuged at 1500 rpm. Pelleted cells were homogenized by sonication in 50 mM Tris-HCl, pH 7.4, containing freshly added protease inhibitors (10 μg/ml phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin). Unbroken cells were removed by centrifugation at $2500g$ for 15 min. Membranes were collected by centrifugation at $45,000g$ for 1 h, then acidified with one volume of 50 mM glycine, pH 3 for 3 min, to dissociate any intracellular loosely bound or adsorbed androgen receptor (Hatzoglou et al., 1994) and resuspended in 10 volumes of Tris-HCl buffer. After an additional centrifugation at $45,000g$ for 1 h, protein concentration was measured by the method of Bradford (1976).

Binding experiments were performed in a final volume of 0.1 ml, containing DU145 cell membranes (2 mg/ml) and 5 nM [^{3}H]testosterone (specific activity 95 Ci/mmol; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) in the absence or presence of different concentrations of dihydrotestosterone or polyphenols, ranging from 10^{-9} to 10^{-6} M. Nonspecific binding was estimated in the presence of 5 μM dihydrotestosterone. After overnight incubation at 4°C, bound radioactivity was separated by filtration under reduced pressure, through GF/B filters, presoaked in 0.5% polyethylenimine in water for 1 h at 4°C and rinsed three times with ice-cold 50 mM Tris-HCl buffer, pH 7.4. Filters were mixed with 3 ml of scintillation cocktail, and the bound radioactivity was counted in a scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) with 60% efficiency for tritium.

Actin Cytoskeleton Staining and Visualization. Cells were grown on poly-L-lysine-coated eight-well chamber slides. After incubation with the different agents for 10 min, the actin networks were visualized by direct fluorescence microscopy. Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.5% Triton X-100 for 15 min, and incubated in blocking buffer (2% BSA in PBS). Actin cytoskeleton was visualized with rhodamine-phalloidin staining (1:400 in PBS containing 0.2% BSA) for 45 min. Specimens were analyzed in a Leica (Wetzlar, Germany) SP confocal microscope.

Signaling Molecule Identification. Testosterone-BSA- or polyphenol-treated cells (10^{-7} M, for the indicated time periods), as well as untreated (control) cells, were washed three times with ice-cold PBS and suspended in cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris, pH 7.4, and 137 mM NaCl, supplemented with protease and phosphatase inhibitors. Cleared lysates were preadsorbed with protein A-Sepharose for 1 h at 4°C and centrifuged, and the supernatants (equal amounts of protein) were subjected to immunoprecipitation using the indicated antibodies and the protein A-Sepharose beads.

For immunoblot analysis, the cell lysates or the immunoprecipitates were suspended in Laemmli's sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membrane and blocked with 5% nonfat dry milk in 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.05% Tween 20 for 1 h at room temperature. Antibody solutions (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.05% Tween 20 containing 5% nonfat dry milk) were added overnight at 4°C (first antibody) and for 1 h (second horseradish peroxidase-coupled antibody). Blots were developed using the enhanced chemiluminescence system, and the band intensities were quantitated by computer-based image analysis (Image Analysis Inc., Ontario, Canada). Antiphosphotyrosine (PY20) and polyclonal antibody for FAK (rabbit) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal anti- PI3K (p85) antibody was purchased from Upstate Biotechnology Inc.

In Vivo Experiments with Nude Mice. Male BALB/c(−/−) nude mice (10 week old) were from Harlan (San Pietro al Natisone, Italy). Animals were injected subcutaneously in the back with 5 × 10^6 DU-145 cells diluted in Matrigel (Sigma) in a total volume of 0.1 ml. After 2 weeks, macroscopic tumors were developed. Then, vehicle (PBS), testosterone-BSA (8 mg/kg), B2 (0.08 mg/kg), or oleylated-B2 (0.16 mg/kg) was used to achieve a calculated concentration of 10^{-7} M of either substance in body fluids. The choice of this concentration derived from in vitro experiments described above. Substances were diluted in PBS and injected intraperitoneally three times per week in a total volume of 0.5 ml. Tumor size was measured with a vernier weekly, and its weight was calculated by the formula $1/2a \times b^2$, where a is the long diameter and b is the short diameter of the tumor (both in cm) (Wang et al., 2003). The animals were sacrificed at the indicated time (4 weeks after the initiation of therapy). Tumors were excised, measured, fixed in formalin, and analyzed by a pathologist. Liver and testes were analyzed by the same pathologist blindly for changes indicative of testosterone or oligomeric proanthocyanidin (OPC) action. The inhibitory rate (IR) of tumor growth was calculated according to the following (Zhou et al., 2005):

$$IR = \frac{C(W_1 - W_0) - T(W_1 - W_0)}{C(W_1 - W_0)}$$

where C is the control group, T is the treated group, W_1 is the tumor weight before treatment, and W_0 is the weight after treatment. The protocol for animal treatment was approved by the University of Crete School of Medicine Research and Ethics Committee.

Histological Staining. Serial sections of tumors (3 μ thick) were cut from each paraffin block (tumors, liver, and testes) and layered on negatively charged (SuperFrost Plus) slides (Kindler GmbH, Freiburg, Germany). One slide was stained with hematoxylin-eosin and observed directly. The labeling streptavidin-biotin method, using the SuperSensitive Biotin-Streptavidin Immunodetection System (QA200-OX; Biogenex, San Ramon, CA) according to the manufacturer's instructions, was used to immunostain sections for mitotic activity with the mouse anti-human monoclonal antibody MIB-1 (M7240; Dako Denmark A/S, Glostrup, Denmark; dilution 1:50). Fast red was used as chromogen, and Mayer's hematoxylin was used for counterstaining. All tumors were analyzed blindly by the same pathologist.

Statistical Analysis. Statistical analysis was performed using the appropriate test and the SPSS computer program (SPSS Inc., Chicago, IL). Statistical significance was set to $p < 0.05$. Chemical fitting was performed with the HyperChem V 8.0.3 program (Hypercube Inc., Gainesville, FL).

Results

Effect of Monomeric and Oligomeric Flavanols on Cell Proliferation of Prostate Cancer Cell Lines. Hormone-sensitive and -resistant prostate cancer cells (LnCaP and DU-145) display a specific membrane binding of nonpermeable testosterone (BSA-conjugated testosterone, testosterone-BSA) as revealed by flow cytometry. In addition, [^{3}H]testosterone binds on acid-stripped prostate cell membranes and can be displaced by testosterone-BSA, with the same affinity as unconjugated analogs (Hatzoglou et al., 2005; Kampa et al., 2006) (Supplemental Fig. 1). These results suggest the existence, in both cell lines, of functional mem-

brane androgen binding sites, independently of the presence of intracellular androgen receptors. In previous studies it was shown that testosterone-BSA, a membrane-acting testosterone analog, decreases cell proliferation of prostate cancer cell lines (Hatzoglou et al., 2005; Kampa et al., 2006), acting through a FAK-PI3K/kt-Cdc42/Rac1-actin signaling pathway (Papakonstanti et al., 2003). On the other hand, proanthocyanidins B2 and B5 were also reported as agonists of membrane androgen binding sites, triggering the same signaling pathway, leading ultimately to a specific actin cytoskeleton redistribution pattern (Nifli et al., 2005). Here, we have performed a systematic analysis of the (4-8)-B-series dimers of catechin and epicatechin, comparing their effects on prostate cancer cell line growth. The derivatives tested are presented in Fig. 1. As shown in Fig. 2, in both hormone-sensitive (LnCaP) and hormone-resistant (DU-145) prostate cancer cell lines, testosterone-BSA (10^{-7} M) induces a significant decrease of cell proliferation, by 25 and 40%, respectively. This result, taking into account the absence of functional intracellular androgen receptors in DU-145 cells, implies a specific action, mediated by membrane androgen sites, which should be distinct molecular entities.

OPCs B1-B4 dispose distinct inhibitory properties in each cell line. In LnCaP cells, the effect of all OPCs was minimal, decreasing cell growth by 7 to 13%. The calculated IC_{50} values for OPCs ranged from 0.5 to 30.4 nM (with B3 = B4 > B2 ≫ B1), whereas for testosterone-BSA it was 0.7 nM. In contrast, in the androgen-insensitive DU-145 cell line, OPCs were more potent than in LNCaP cells, decreasing cell growth from 12 to 31%. IC_{50} values ranged from 0.6 to 74 nM, with B2 ≫ B3 = B4 > B1 (Table 1). In addition, the inhibitory effect of testosterone-BSA in DU-145 cells was more pronounced (~40%) with an IC_{50} value similar to the one observed in LnCaP cells (0.8 nM).

Implication of Galloylation in Oligomeric Flavanol Activity Modification. Previous data showed that galloyl-

esters of monomeric catechins exert a substantial effect on cell survival and metabolism (see Butt and Sultan, 2009 for review). Here, we have investigated the effects of galloylated epicatechin monomer and the 3-O-galloyl esters of dimers B1 and B2. Data are presented in Table 1 and Fig. 2B. Galloylation did not alter the potency of epicatechin monomer on DU145 cells, whereas it partially reversed its action on the LnCaP cell line. In contrast, it markedly enhanced the effect of B1 on LnCaP cells, whereas it completely annihilated its action in DU-145 cells. B2 galloylation slightly increased its effect on LNCaP cells; however, it significantly potentiated growth inhibition of DU-145 cells, shifting IC_{50} values from 0.6 to 45.3 nM. We therefore concluded that galloylation could not uniformly affect the antiproliferative activity of the oligomeric flavanols and could not be of any value in prostate cancer, especially in the advanced, hormone-resistant, stages of the disease. In addition, these data indicate that inhibition of binding of dimeric flavanols to the putative androgen site on the plasma membrane might occur through steric hindrance galloylation at position 3.

Novel Oleylated OPC Derivatives Exert a Potent Antiproliferative Effect on Prostate Cancer Cell Growth. Among tested natural OPCs, B2 flavanol dimer exerted the more pronounced antiproliferative action in DU145 cells, in line with previous data. We further investigated structural similarities between B2 and testosterone that could account for common actions. Molecular simulation revealed that the best fit of testosterone and B2 depends on hydroxyl moieties at positions 3 and 4 of ring B and position 5 of ring D (or alternatively positions 3 and 4 of ring E and position 5 of ring A) of B2 and oxygen at position 3, hydroxyl at position 17, and methyl at position 13 of the testosterone molecule (root mean square 1.02 Å; Supplemental Fig. 2). Testosterone-BSA (see Fig. 1) bears a carboxyl-methyl-BSA substitution at position 3 that could account for the observed differences in B2 and testosterone-BSA IC_{50} values. However, according to our pre-
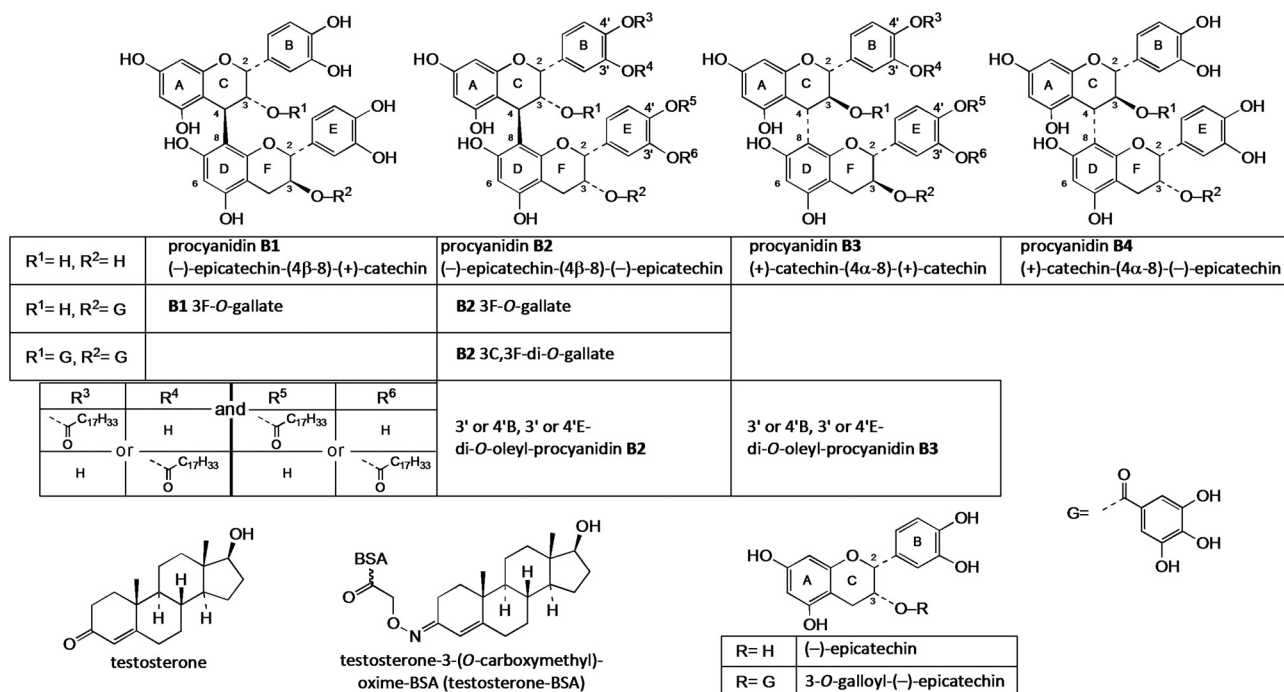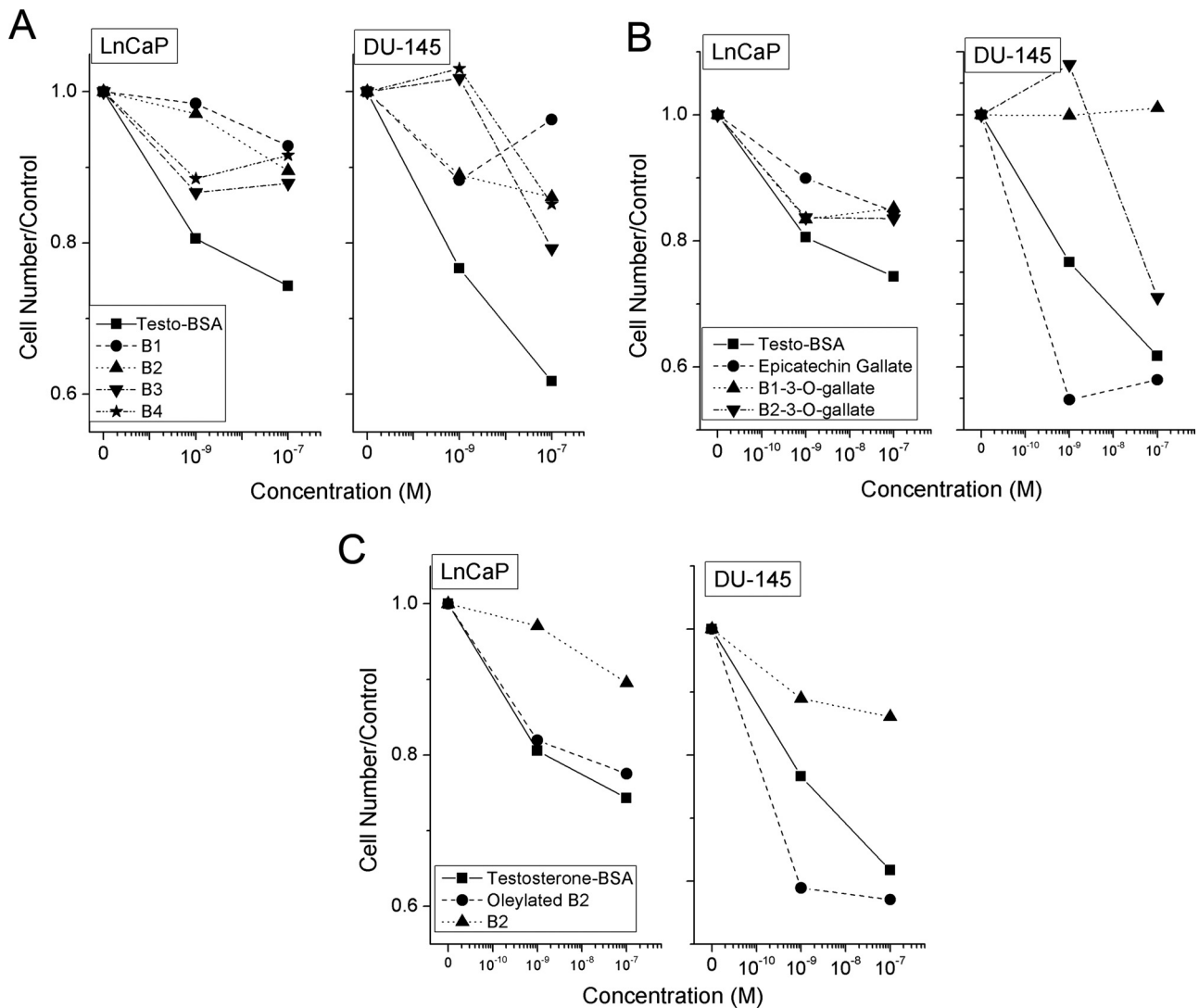

Fig. 1. Chemical structures of the substances used in the study.

Fig. 2. Effect of OPCs B1-B4 (A), galloylated derivatives (B), and dioleylated B2 (C) on the growth of androgen-sensitive (LnCaP) and androgen-resistant (DU145) human prostate cancer cells. Normalized results compared with control (nontreated) cells are presented. The effect was measured after two cell cycles. Mean is of three different experiments performed in triplicate.

vious results, the carboxymethyl-oxime group did not originate the observed actions of testosterone-BSA (Kampa et al., 2002; Papakonstanti et al., 2003). In an attempt to improve B2 activity and bioavailability, we synthesized oleic acid ester derivatives. The dioleylated B2 derivative exerted a

very potent inhibitory effect on cell growth in LnCaP cells and especially in DU-145 cells, with an IC_{50} value similar to that of testosterone BSA (Table 1). We have therefore focused on this B2-diester, trying to investigate its mode of action, both in vitro and in vivo. Similar, albeit less pronounced,
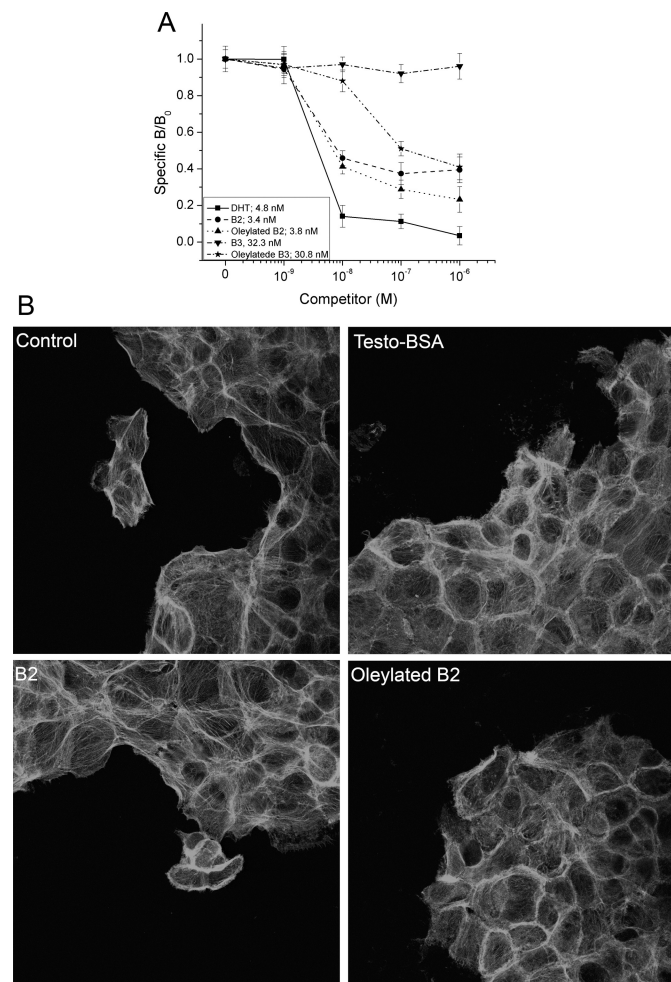
TABLE 1
Maximal inhibition (normalized as per control-untreated cells) and IC_{50} values of substances used in the study on cell proliferation of prostate cancer cell lines (mean ± S.E.M. of three independent experiments, performed in triplicate)

	LnCaP		DU-145	
	Maximum Inhibition	IC_{50}	Maximum Inhibition	IC_{50}
		nM		*nM*
Testosterone-BSA	0.74 ± 0.06	0.7 ± 0.05	0.62 ± 0.08	0.8 ± 0.07
B1	0.93 ± 0.06		0.88 ± 0.03	74.3 ± 0.06
B2	0.9 ± 0.02	30.4 ± 3.54	0.87 ± 0.06	0.6 ± 0.02
B3	0.87 ± 0.04	0.5 ± 0.01	0.79 ± 0.11	47.4 ± 6.87
B4	0.88 ± 0.03	0.5 ± 0.04	0.85 ± 0.04	48.1 ± 4.88
Epicatechin	0.40 ± 0.03	0.5 ± 0.07	0.58 ± 0.04	0.5 ± 0.04
Epicatechin-3-*O*-gallate	0.86 ± 0.04	0.7 ± 0.03	0.55 ± 0.04	0.4 ± 0.02
B1–3F-*O*-gallate	0.83 ± 0.02	0.5 ± 0.05	1	
B2–3F-*O*-gallate	0.84 ± 0.02	0.5 ± 0.03	0.71 ± 0.07	45.3 ± 7.28
Oleylated B2	0.78 ± 0.05	0.6 ± 0.03	0.57 ± 0.05	0.4 ± 0.03
Oleylated B3	0.79 ± 0.06	0.65 ± 0.04	0.74 ± 0.03	35.4 ± 6.21

effects were also detected with the corresponding oleyl ester derivative of B3 dimer (Table 1).

Interaction of Oleylated Oligomeric Flavanols with Membrane Androgen Sites and Actin Cytoskeleton. Previous data indicate that B2 competes for binding on membrane androgen sites and induces changes similar to that of testosterone on actin cytoskeleton, through activation of the same intracellular signaling pathways (Nifli et al., 2005) (Supplemental Fig. 3). Here, we compared the affinity of oleylated B2 on membrane androgen binding sites (Fig. 3A) in DU-145 prostate cancer cell membranes, a cell line not expressing functional intracellular androgen receptors and presenting an enhanced membrane testosterone binding. As shown, B2 expresses an IC_{50} value for testosterone displacement compatible to that of dihydrotestosterone and its effect on cell growth (3.4 nM). This affinity is not significantly modified by its oleylation, although this esterification leads to an increase in its potency to displace [3H]testosterone. It is noteworthy that for B3 in the same system and at the studied range a very low affinity was found. This is in accordance with its low antiproliferative action.

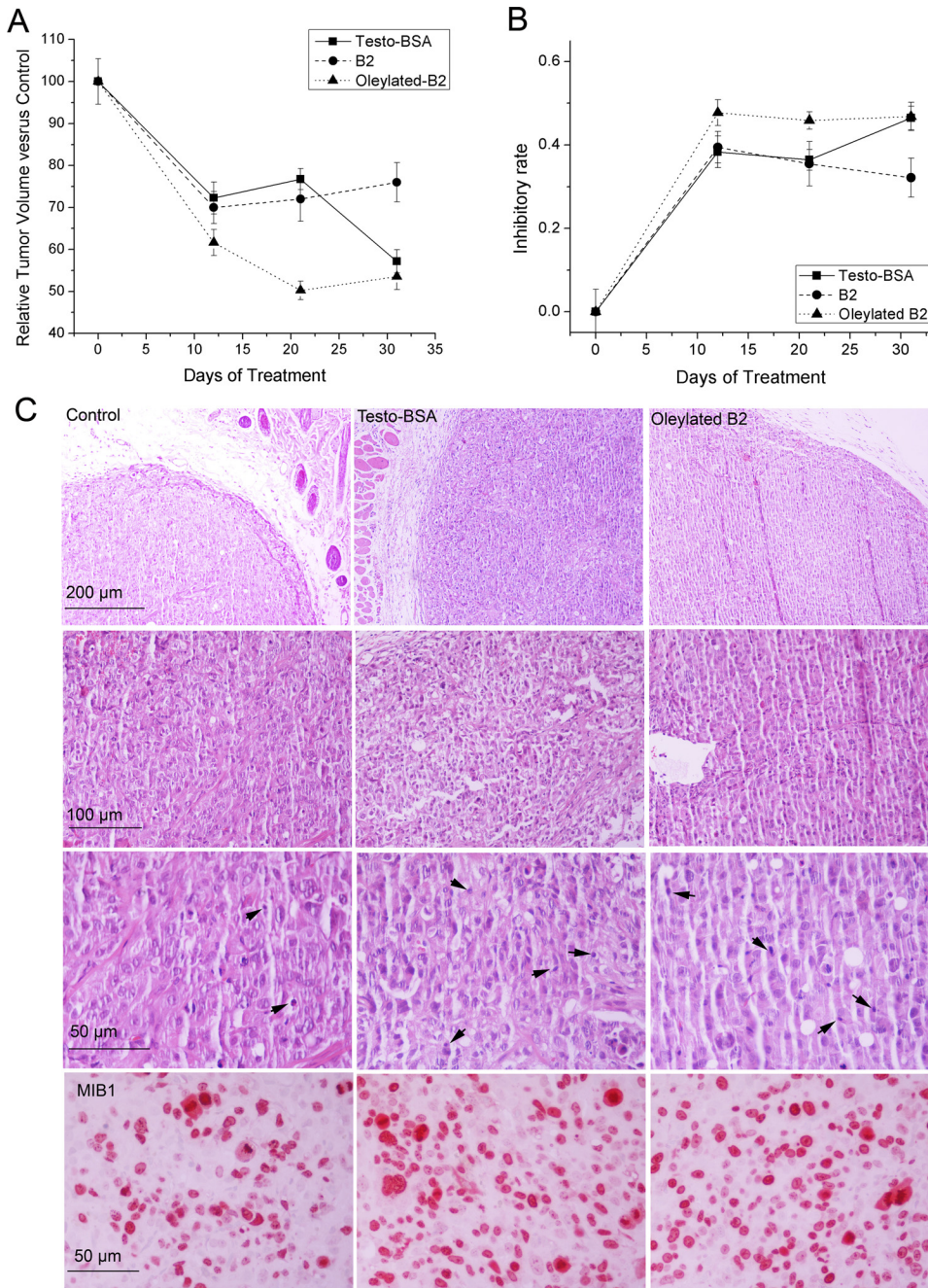In previous works (Papakonstanti et al., 2003; Nifli et al.,

2005), we have reported that both testosterone and B2 modify the same intracellular signaling cascades, namely FAK-PI3K/Akt-Cdc42/Rac1, leading to actin submembrane redistribution. Here, we show that, in addition to membrane androgen binding and signaling molecule activation (Supplemental Fig. 3), both B2 and oleylated B2 induced a peripheral actin redistribution, as obtained with testosterone-BSA (Fig. 3B). These data suggest that oleylated B2 might trigger similar changes as testosterone-BSA and nonoleylated B2.

Effect of Oleylated or Native Proanthocyanidins on the Regression of Prostate Tumor Xenografts in BALB/c(−/−) Mice. The in vitro results so far suggest that both B2 and its oleylated conjugate exert similar effects on testosterone-BSA, acting on membrane androgen sites. We have further verified these actions in vivo. BALB/c(−/−) mice were inoculated with DU145 cells. We used this cell line because: 1) it contains no functional intracellular androgen receptors, permitting the deciphering of the net effect of membrane androgen sites; 2) the effect of both testosterone and B2 analogs on this cell line is maximized, compared with the LnCaP cells; and 3) a possible positive effect of B2 analogs should provide a hint for their potential use in advanced prostate cancer therapy. After tumor growth (~15 days later), mice were treated with vehicle (PBS), testosterone-BSA (8 mg/kg), B2 (0.08 mg/kg), or oleylated-B2 (0.16 mg/kg), resulting in a calculated concentration of 10^{-7} M of either agent in body fluids. Substances were administered intraperitoneally three times per week for 1 month. Tumor size, calculated according to the formula presented under *Materials and Methods*, was measured every 10 days. Results, presented in Fig. 4A, show that testosterone-BSA decreased tumor size by ~40%, in accord with our previously published data (Hatzoglou et al., 2005; Kampa et al., 2006). B2 decreased tumor size by ~30%, whereas oleylated-B2 had the maximal effect, decreasing tumor size by ~50%. When expressing results as the inhibitory rate of each substance (Zhou et al., 2005) the early and sustained effect of oleylated-B2 becomes obvious (Fig. 4B). This is accompanied by a change in cell morphology of the tumors (Fig. 4C); tumor cells of treated animals were smaller, presenting an increased apoptosis, as evaluated by the higher rate of cells displaying characteristic morphological features that are manifest even in routinely stained sections, such as shrinkage, intense eosinophilic cytoplasm, pycnotic nuclei, and increased amount of apoptotic bodies (control 2 apoptotic cells per high-power field; testosterone-BSA 4/field and oleylated B2 4/field, $p < 0.01$; Fig. 4C, arrows). In parallel, we assayed a morphologic evaluation of the proliferation status of treated tumor cells, by means of the mitotic index, which is defined as the ratio between the number of cells in mitosis and the total number of cells. Practically, with light microscopy in histologic slides routinely stained by hematoxylin/eosin, the mitotic index can be worked out as the number of cells containing visible chromosomes divided by the total number of cells in the field of view. In addition, a more refined identification of cells in the cell cycle was achieved by means of the MIB1 antibody, which is raised against the nuclear antigen Ki67. The histologic analysis of untreated tumors showed mitoses predominating at the periphery of the tumors, namely their "growing edge," verifying previous data in the same strain, using testosterone-BSA conjugates (Kampa et al., 2006). The evaluation of mitotic index revealed a decrease of proliferative cells in testosterone- and

Fig. 3. A, displacement of [3H]testosterone from membrane binding sites of DU145 cell membranes by dihydrotestosterone (DHT), B2, B3, and their oleylated conjugates. Shown is means ± S.E.M. of three different experiments performed in triplicate. B, modification of the actin cytoskeleton cellular distribution in testosterone-BSA-, B2-, and oleylated B2-treated DU145 cells. Typical sections are presented.

Fig. 4. In vivo effects of oleylated B2 in the reduction of BALB/c($-/-$) DU145 tumors. Ten-week-old male BALB/c($-/-$) animals were injected with 5×10^6 DU145 cells in Matrigel. After the development of tumors, animals were injected with PBS (control), testosterone-BSA (8 mg/kg), B2 (0.08 mg/kg), or oleylated-B2 (0.16 mg/kg), providing a concentration of 10^{-7} M of either substance in body fluids. A and B, tumor volume (A) and inhibitory rate (B) are calculated as described under *Materials and Methods*. C, typical histological sections of the tumors are shown, in different magnifications, stained with hematoxylin and eosin, and MIB1 antibody staining Ki67. Apoptotic cells are denoted by arrows in high-magnification sections.

oleylated B2-treated tumor cells, confirming our in vitro data.

Oleylated OPC Does Not Exert Toxicity or Androgenic Actions. Although previous data suggest that testosterone-BSA does not release free testosterone in body fluids (Hatzoglou et al., 2005), in the present work we have assayed, using a specific testosterone assay, its concentrations in the plasma of treated mice. No significant changes between control and testosterone-treated mice were observed (not shown). To access the possible proandrogenic effect of B2, we have analyzed the volume and histology of testes. Testicular volume remained unaltered in treated animals, compared with control (not shown), whereas histology of the testes did not show any modification of spermatozoide production and maturation (Supplemental Fig. 4).

In addition, because the liver is the main organ of detoxification, we have assayed liver aminotransferases (alanine aminotransferase, aspartate aminotransferase) through specific assays in serum. No modification of enzyme levels was observed (not shown). In addition, liver morphology did not differ in treated animals compared with the control (Supplemental Fig. 3). We have therefore concluded that neither testosterone-BSA nor oleylated B2 exert any significant toxicity in treated mice after 1 month of treatment.

Discussion

Prostate cancer is the most common diagnosed neoplastic disease in Western men and the second-leading cause of cancer-related deaths. The natural history of the disease

usually starts as an androgen-dependent tumor and evolves to androgen insensitivity. Current treatment protocols suggest surgical ablation of the tumor, followed by antiandrogen and/or chemotherapy, although reoccurrence is not excluded and, especially at the advanced stages of the disease, the outcome is poor. Therefore, alternative therapeutic approaches are being investigated.

Our previous data indicate that human prostate cancer specimens express preferentially membrane androgen binding sites compared with adenomas or nontumorous tissue, which correlate with the severity of the disease, expressed by the Gleason's score (Dambaki et al., 2005). In addition, membrane androgen receptor agonists (in the form of testosterone-protein conjugates), alone (Hatzoglou et al., 2005) or in association with taxane-based drugs (Kampa et al., 2006), induce the regression of prostate cancer xenografts (both hormone-sensitive or -resistant), proposed as an alternative therapeutic strategy. However, steroid-protein conjugates are difficult to manage, necessitating intravenous administration. In the search for potential micromolecular agonists of membrane androgen sites, we have previously reported that OPC of the B series could interact with these sites (Nifli et al., 2005). Here, we analyzed natural OPC B-series and provide evidence about a specific effect of a novel, semisynthetic, oleylated derivative.

Although estrogenic actions have been studied extensively for a number of polyphenols (see Kampa et al., 2007 for an extensive review), polyphenols' androgenic activity is rather rarely assayed. Previous data have shown that catechins may interact with androgen receptors in prostate cancer cell lines (Kampa et al., 2000), while they may modulate androgen secretion (Liao, 2001) and the expression of androgen receptors (Ren et al., 2000). A study further suggests a potential membrane-initiated action of catechin analogs (Bastianetto et al., 2009), rapidly modifying protein kinase C isoforms. Our previous results (Nifli et al., 2005) and data presented here suggest that catechins interact with membrane androgen sites. In addition, our data support that OPC derivatives (and especially B2, which has the highest activity) present a spatial conformation similar to that of testosterone, providing a molecular hint of their interaction with androgen sites and androgen-like actions. In addition, we report that OPC derivatives compete for binding, induce signaling molecule changes, modify actin cytoskeleton, decrease cell growth, and reduce tumor size in mice in a way similar to that of conjugated testosterone. In that respect we propose them to be agonists of membrane androgen sites, an element that might be of potential therapeutic importance.

The nature of membrane androgen sites has not been elucidated until now. Their characterization involves pharmacological elements, namely the binding of androgen to membranes (Benten et al., 1999), the triggering of specific signaling cascades (Pelekanou et al., 2010), the modification of intracellular elements (Papakonstanti et al., 2003), the movements of ions through plasma membrane (see Kaarbø et al., 2007 for a review), and the transcriptional regulation of a number of genes different from those initiated from unconjugated testosterone (Notas et al., 2010). There is evidence that they might be G-coupled protein receptors (Kaarbø et al., 2007), intracellular receptors anchored to the plasma membrane through post-transcriptional modifications (Pedram et al., 2007), ion channels (Kelly and Levin, 2001), or

totally new, not-yet-identified, proteins. Our data provide some new clues to this discussion. Indeed, we show that testosterone binding and actions on both androgen receptor-bearing (LnCaP) and/or androgen receptor-deprived (DU-145) cells are similar, suggesting the molecular heterogeneity of membrane and intracellular androgen sites.

Not all studied OPC derivatives exert a specific membrane-initiated androgenic effect. Focusing on DU-145 cells (which are androgen-insensitive) and the molecular structures presented in Fig. 1, it becomes obvious that some specific molecular elements are needed for such an effect: 1) the β configuration of the 4-8-link between the two flavanol moieties, 2) the "2,3-*cis*" configuration (epicatechin 2*R*,3*R*-series) of the monomers (especially the α configuration of the hydroxyl group at position 3F), and 3) the presence of a free hydroxyl group at this 3F position. In addition, oleylation confers an increased affinity to the OPC molecule. It is not clear why this latter modification increases the potency of OPC molecules. One possibility might be that oleylation confers to the molecule a structure similar to that of conjugated testosterone, but more certainly, this is related to the increased lipophilicity of the resulting molecule, permitting its better incorporation into the plasma membrane and a better interaction with growth factor receptors (an element described for membrane-acting androgen; see Kampa et al., 2008 for a review) or with membrane-anchored, conventional (Marino and Ascenzi, 2006), or not, androgen sites. Indeed, the latter hypothesis has been explored in the case of estrogen receptors (ERs), where an alternative form of ERα (ERα36) has been proposed to initiate the membrane effects of the hormone (Wang et al., 2005).

The effect of B2, especially oleylated-B2, is not restricted to prostate cancer cells in vitro, but extended also in vivo to BALB/c(−/−) mice xenografted with DU145 human prostate cancer cells. B2, and especially oleylated-B2, exerts a 50% inhibitory effect on tumor growth in animals that have already developed tumors, a situation resembling prostate cancer in humans. This growth inhibition implies a double effect, targeting proliferation and apoptotic potential of prostate cancer cells, as demonstrated in morphological evaluation of histological sections. This is not surprising, because, in essence, tumor growth is the net result of cell proliferation and cell loss. On the other hand, the proapoptotic effect in DU145 xenografts gains additional importance if we take under consideration that these cells display an enhanced resistance to apoptosis. Indeed, through the prostate carcinoma progression, there is a trend to gain resistance to the apoptosis-inducing hormone withdrawal. High bcl-2 expression is found in androgen-independent prostate tumors, whereas the extent of apoptosis was found to be lower in recurrent than primary tumors (Soini et al., 1998). In this perspective, our data suggest that OPCs, and especially some of their novel oleylated derivatives, are probably the first small-molecule agonists of membrane androgen sites. They could be useful novel agents for the advanced treatment of prostate cancer, alone or in combination with cytoskeletal-acting drugs (Kampa et al., 2006). This is further corroborated by their small molecular weight, the fact that they might be administered orally (because significant concentrations have been detected in biological fluids after OPC-rich foods; Manach et al., 2005), and the absence of androgenic or (liver) toxicity as reported here.

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Novel oligomeric proanthocyanidin derivatives interact with membrane androgen sites and induce regression of hormone-independent prostate cancer

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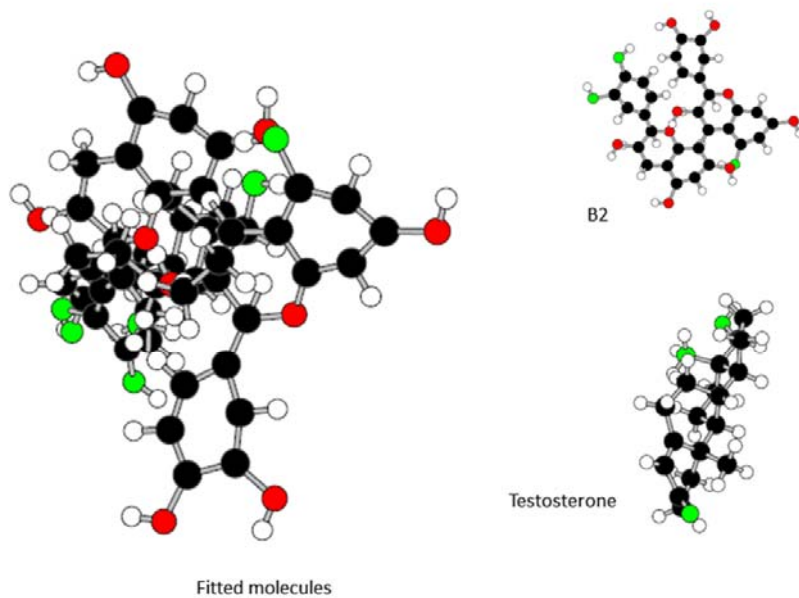
Supplemental Figure 1

Flow cytometry (left panels) and saturation binding (right panels) detection of membrane androgen binding sites on LnCaP (upper panels) and DU-145 prostate cancer cells (lower panels). Cells (10^6 cells/mL in PBS) were incubated with 1 μM ERα17p-FITC or estradiol-BSA-FITC conjugates, for 10 min, in absence or in presence of unlabeled ERα17p (10 μM). BSA-FITC was used to determine non-specific binding. At least 10,000 gated cells were analyzed and fluorescence was measured at a Becton-Dickinson FACSCalibur apparatus (Becton-Dickinson, Franklin Lakes, NJ) and analyzed with the CellQuest-pro (Beckton Dickinson) software. For binding experiments, membranes were isolated, acidified and processed for saturatiuon binding, as described in the Material and Methods section. Inserts present analysis of satruration binding data in Scatchard coordinates.
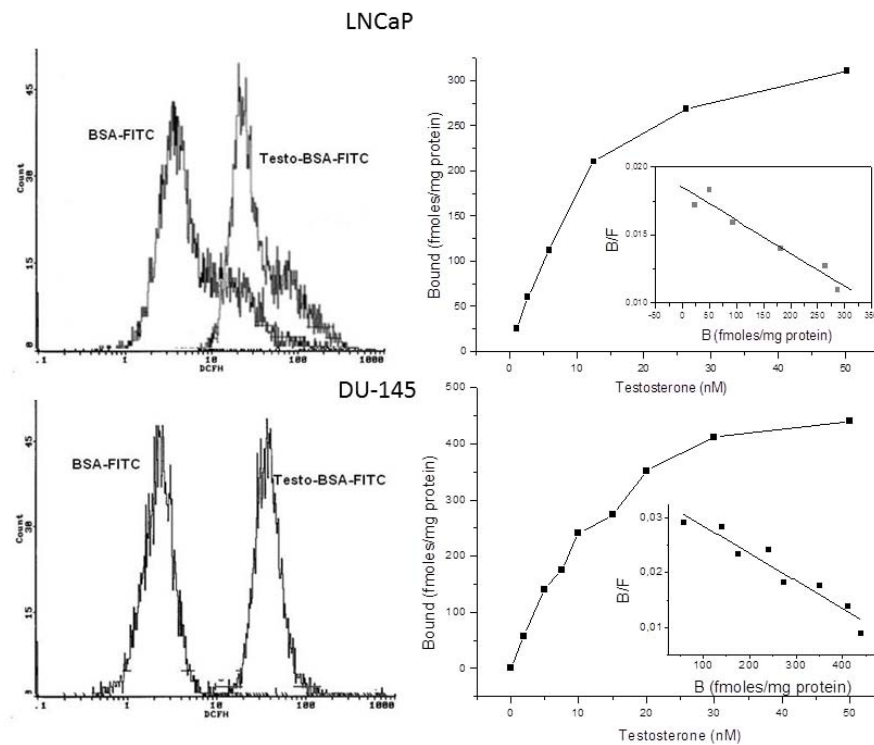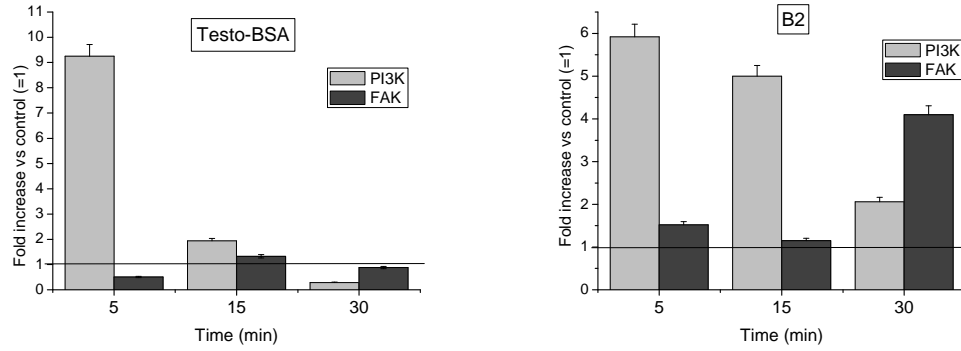
Supplemental Figure 2

Simulation of the spatial conformation of testosterone and B2 OPC. (carbon: black spheres; oxygen: red spheres; hydrogen: white spheres). Green spheres represent the moieties on which molecular similarities were calculated and molecules overlapped.

B2

Testosterone

Fitted molecules

Supplemental Figure 3

Time course of the phosphorylation state of PI3K and FAK, after incubation of cells with 10^{-7} M testosterone-BSA or B2 for the indicated time periods (5, 15, 30 min). DU-145 cells were lysed and the phosphorylated PI3K and FAK were assayed by electrophoresis and immunoblotting as described in Materials and methods. Data Mean±SEM of three different experiments) are presented as compared to the corresponding control, set as one.

Supplemental Figure 4

Liver and testes have been withdrawn from treated mice (after 4 weeks treatment with oleylated B2, testosterone-BSA or vehicle), fixed in formol, and sections were stained with hematoxylin-eosin and examined. As shown, no morphological changes were apparent. Testes did not show any androgenic effect (increased spermatogenesis).