JPET Fast Forward. Published on January 4, 2011 as DOI:10.1124/jpet.110.177246

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

Marilena Kampa, Katerina Theodoropoulou, Fani Mavromati, Vassiliki Pelekanou, George Notas, Eleni D. Lagoudaki, Artemissia-Phoebe Nifli, Cécile Morel-Salmi, Efstathios N. Stathopoulos, Joseph Vercauteren, Elias Castanas

Laboratories of Experimental Endocrinology (MK, KT, FM, VP, GN, APN, EC) and Pathology (EDL, ENS), University of Crete, School of Medicine, Heraklion, Greece

Polyphenols R&D SARL (CMS), 301 Chemin de Caylus 34170 Castelnau le Lez, France

Laboratory of Pharmacognosy (JV), School of Pharmacy, University of Montpellier I, France

Running Title: Oleylated B2 OPC in prostate cancer management

Corresponding author: Elias Castanas, Laboratory of Experimental Endocrinology, University of Crete, School of Medicine, P.O. Box 2208, Heraklion, 71003, Greece, Tel: +30 2810 394580, Fax: +30 2810 394581, e-mail: castanas@med.uoc.gr

Document statistics:

Number of text pages: 14

Number of tables: 1

Number of Figures: 4

Number of references: 40

Number of words: Abstract: 230

Introduction: 603

Discussion: 1075

Non-standard abbreviations: OPC: oligomeric proanthocyanidins

Recommended section assignment: Drug Discovery and Translational Medicine

ABSTRACT

Prostate cancer is the commonest male malignancy in Western societies and current therapeutic approaches are evolving to manage growth, recurrence and mortality neoplasia. Recently, membrane androgen receptors (mAR) were characterized in human prostate cancer, being preferentially expressed in tumor than in benign gland areas. Furthermore, mAR agonists (protein-conjugated testosterone) decrease *in vitro* prostate cancer cell growth and induce apoptosis, while *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. Seeking for new small molecules ligands of mAR, we report that flavanol dimers B1-B4 (oligomeric procyanidins, OPC) decrease *in vitro* growth of the androgen-sensitive (LnCaP) and 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-BSA conjugate. Galloylation does not confer an additional advantage; however, oleylation increases their antiproliferative potency by a factor of 100. In addition, we report that B2, oleylated or not, displaces testosterone from mAR with an IC_{50} at the nM 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 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 integrating 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 later lead ultimately 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. Non-mutually exclusive possibilities include: (i) membrane anchoring of intracellular receptors through post-translational modifications, acting independently or in association with growth factor receptors (Marino and Ascenzi, 2006); (ii) truncated or alternative spliced steroid receptors (Wang et al., 2006); (iii) novel receptor proteins (Zhu et al., 2003). Indeed, a number of receptors have been reported, belonging mainly to the GPCR family, to mediate some of the membrane-initiated steroid effects. However, there is an extensive discussion whether these receptors are true steroid receptors or co-receptor proteins (Levin, 2009).

Even if the identity of androgen membrane receptor(s) 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), as well as in breast (Pelekanou et al., 2007), prostate (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 to modify a number of cellular responses, including signaling molecules, enzymes, and/or transcription factors, leading ultimately to modification of the cell fate, towards survival or apoptosis (see Kampa et al., 2002, for a review). Interestingly, a recent report suggests also a potential membrane-initiated action of catechin analogs (Bastianetto et al., 2009), interacting with PKC 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 and PI3-K, modifying further actin polymerization, 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.

MATERIAL AND METHODS

Cell lines and culture conditions

LNCaP and DU-145 cells (DSMZ, Braunschweig, Germany), were cultured in RPMI 1640 medium (Gibco-Invitrogen, Paisley, UK), 10% fetal bovine serum (FBS) at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. Testosterone-3-(*O*-carboxymethyl)-oxime–BSA (10 molecules 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 DCC treatment (dextran 0.05mg/ml and charcoal 50mg/ml) for 30 min, in order 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 EtOAc grape seeds extracts (*Vitis vinifera*, Vitaceae) on which Centrifugal Partition Chromatography (CPC) 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 (Prep-HPLC) of each blocks, using a 0-100% methanol-water gradient. Eleven proanthocyanidins were thus isolated as pure compounds and fully characterized. Their identification was ascertained by two complementary methods: 1) by comparing 2D long range NMR spectra (Gradient Accelerated Spectroscopy-HMBC (Bax and Summers, 1986)) of

peracetylated proanthocyanidins with those of references (Balas et al., 1995), to establish their gross structure and 2) by comparing chromatographic behaviour 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 co-injection on an analytical HPLC system, coupled to PDA-detection online with an electrospray ionization mass spectrometer, to confirm the nature (catechin or épicatéchine series) of each moiety and to ascertain the type of the inter-flavanolic linkage (IFL). HPLC conditions for analysis of proanthocyanidins (method 1) were: Column Synergi 4 hydro – RP 80A (250x2.0 mm) from Phenomenex; water-0.0025 % TFA (v/v, solvent A), methanol-0.0025% TFA (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 (4.6x250mm) from Waters, 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. ESI-MS is a LCQ Advantage from ThermoFinnigan, monitored by Xcalibur 2.1 package software.

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), procyanidin B2 3C,3F-di-*O*-gallate (R_t= 33.0 min). These proanthocyanidins were already 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 $CHCl_3$, was added 766 □l 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 $CHCl_3$ was added dropwise over a 2 hours period. The $CHCl_3$ used was checked to be free of any trace of EtOH (stabilized by amylene). The mixture was stirred at room temperature under nitrogen for 6 additional hours. Sodium bicarbonate aqueous solution was then added until the pH was made alkaline. A saturated solution of NH_4Cl was added, prior the extraction by $CHCl_3$. The organic layer was washed with water, dried over sodium sulphate, 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 ESI-MS in the negative ionization mode exhibited a signal of pseudomolecular ion $[M-H]^-$ in favour 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). While NMR clearly confirmed the presence of two oleyl residues, it was not possible to unambiguously determine which phenolic group(s) 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 PMSF and 1 μg/ml aprotinin). Unbroken cells were removed by centrifugation at 2500g for 15min. Membranes were collected

by centrifugation at 45,000g for 1 hour, then acidified with one volume of 50 mM glycine pH 3 for 3 min, in order to dissociate any intracellular loosely bound or adsorbed androgen receptor (Hatzoglou et al., 1994), and resuspended in ten volumes Tris-HCl buffer. After an additional centrifugation at 45,000g for 1 hour, protein concentration was measured by the method of Bradford.

Binding experiments were performed in a final volume of 0.1 ml, containing DU145 cell membranes (2 mg/ml) and 5nM of [^{3}H]testosterone (specific activity 95 Ci/mmole, Amersham-Pharmacia, Buckinghamshire, UK) in the absence or in the presence of different concentrations of dihydrotestosterone (DHT) or polyphenols, ranging from 10^{-9} to 10^{-6} M. Non-specific binding was estimated in the presence of 5μM DHT. After overnight incubation at 4^{o}C, bound radioactivity was separated by filtration under reduced pressure, through GF/B filters, pre-soaked in 0.5% polyethylenimine (PEI) in water for 1 hour at 4 ^{o}C and rinsed three times with ice-cold 50 mM Tris-HCl buffer pH 7.4. Filters were mixed with 3 ml scintillation cocktail and the bound radioactivity was counted in a scintillation counter (Perkin Elmer, Foster City, CA) with 60% efficiency for Tritium.

Actin cytoskeleton staining and visualization

Cells were grown on poly-L-lysine-coated 8 well chamber slides. After incubation with the different agents for 10 min, actin network were visualized by direct fluorescence microscopy. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes 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 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, 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-PAGE. Proteins were transferred onto nitrocellulose membrane, and blocked with 5% nonfat dry milk in TBS-T (20 mM Tris pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 1h at room temperature. Antibody solutions (in TBS-T containing 5% nonfat dry milk) were added overnight at 4 °C (first antibody) and for 1h (second horseradish peroxidase-coupled antibody). Blots were developed using the ECL system and the band intensities were quantitated by PC-based image analysis (Image Analysis Inc., Ontario, Canada). Anti-phosphotyrosine (PY20) as well as polyclonal antibody for FAK (rabbit) were from Santa Cruz Biotechnology Inc. Rabbit polyclonal anti-PI-3 kinase(p85) antibody was purchased from Upstate Biotechnology Inc.

***In vivo* experiments with nude mice**

Male BalbC$^{-/-}$ nude mice (10 week old) were from Harlan (Italy). Animals were injected subcutaneously in the back with $5x10^6$ 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), in order 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 3 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 OPC action. The inhibitory rate (IR) of tumor growth was calculated according to the following equation 1 (Zhou et al., 2005):

$$IR = \frac{C\,(W_1-W_0) - T\,(W_1-W_0)}{C\,(W_1-W_0)} \qquad \text{Equation 1}$$

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

Histological staining

Serial sections of tumors (3μ thick) were cut from each paraffin block (tumors, liver and testes) and layered on negatively charged (SuperFrost Plus) slides (Kindler O 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, dilution 1:50). Fast red was used as chromogen, and Mayer's hematoxylin for counterstaining. All tumors were analyzed blindly by the same pathologist.

Statistical analysis

Statistical analysis was performed by the use of appropriate test, using the SPSS (SPSS, Chicago, IL) computer program. 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 non-permeable testosterone (BSA-congugated testosterone, testostrerone-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, and Supplemental Figure 1). These results suggest the existence, in both cell lines, of functional membrane 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/Akt-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 lines growth. The derivatives tested are presented in Figure 1. As shown in Figure 2, in both hormone-sensitive (LnCaP) and 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 7-13%. The calculated IC_{50}s for OPCs ranged from 0.5 to 30.4 nM (with B3=B4>B2>>B1), while for testosterone-BSA 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 12-31%. IC_{50}s ranged from 0.6-74 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} similar to the one observed in LnCaP cells (0.8 nM).

Implication of galloylation in oligomeric flavanols 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 Figure 2B. Galloylation did not alter the potency of epicatechin monomer on DU145 cells, while it partially reversed its action on LnCaP cell line. In contrast, it markedly enhanced the effect of B1 on LnCaP cells, while 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} from 0.6 nM to 45.3 nM. We therefore concluded that galloylation could not uniformly affect the antiproliferative activity of the oligomeric flavanols and that it 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 probably, 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, 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 (RMS 1.02Å, Supplemental Figure 2). Testosterone-BSA (see Figure 1) bears a carboxy-methyl-BSA substitution at position 3 that could account for the observed differences in B2 and testosterone-BSA IC_{50}s. However, according to our previous 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 both LnCaP and especially in DU-145 cells, with an IC_{50} similar to that of testosterone BSA (Table 1). We have therefore focused at this B2-diester, trying to investigate its mode of action, both *in vitro* and *in vivo*. Similar, albeit less pronounced 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) and supplemental Figure 3. Here, we compared the affinity of oleylated B2 on membrane androgen binding sites (Figure 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} 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 [^{3}H]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 (Nifli et al., 2005; Papakonstanti et al., 2003), we have reported that both testosterone and B2 modify the same intracellular signaling cascades, namely FAK-PI3K/Akt-Cdc42/Rac1, leading to actin sub-membrane redistribution. Here, we show that, in addition to membrane androgen binding and signaling molecule activation (Supplemental Figure 3), both B2 and oleylated B2 induced a peripheral actin redistribution, as obtained with testosterone-BSA (Figure 3B). These data suggest that oleylated B2 might trigger similar changes as testosterone-BSA and non-oleylated B2.

Effect of oleylated or native proanthocyanidins on the regression of prostate tumor xenografts in BALBc$^{-/-}$ mice

The *in vitro* results so far suggest that both B2 and its oleylated conjugate exert similar effects to testosterone-BSA, acting on membrane androgen sites. We have further verified these actions *in vivo*. BALBc$^{-/-}$ mice, were inoculated with DU145 cells. The reasons of using this cell line were: (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, as compared to the LnCaP cells; (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 to a calculated concentration of 10^{-7}M of either agent in body fluids. Substances were administered intraperitoneally, three times per week for one month. Tumor size, calculated according to the formula presented in the Material and Methods section, was measured every 10 days. Results, presented in Figure 4A show that testosterone-BSA decreased tumor size by ~40%, in accord to our previously published data (Hatzoglou et al., 2005; Kampa et al., 2006); B2 decreased tumor size by ~30%, while oleylated-B2 had the maximal effect, decreasing tumor size by ~50%. Expressing results as the inhibitory rate of each substance (Zhou et al., 2005) the early and sustained effect of oleylated-B2 becomes obvious (Figure 4B). This is accompanied by a change in cell morphology of the tumors (Figure 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, like 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, $p<0.01$, Figure 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 effected 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 evidenced a decrease of proliferative cells in testosterone- and 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). In order to access the possible pro-androgenic effect of B2, we have analyzed the volume and histology of testes. Testicular volume remained unaltered in treated animals, as compared to control (not shown), while histology of the testes did not show any modification of spermatozoide production and maturation (Supplemental Figure 4).

In addition, as the liver is the main organ of detoxification, we have assayed liver aminotransferases (ALT, AST) through specific assays, in serum. No modification of enzyme levels was observed (not shown). In addition, liver or testes morphology did not differ in treated animals, as compared to the control (Supplemental Figure 3). We have therefore concluded that neither testosterone-BSA nor oleylated B2 exert any significant toxicity in treated mice, after one month treatment.

DISCUSSION

Prostate cancer is the commonest diagnosed neoplastic disease in Western male populations 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 anti-androgen and/or chemotherapy, though reoccurrence is not excluded and, especially at the advanced stages of the disease, the outcome is poor. Therefore, alternative therapeutic approaches are investigated.

Our previous data indicate that human prostate cancer specimens express preferentially membrane androgen binding sites, as compared to adenomas or non-tumorous 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 of potential micromolecular agonists of membrane androgen sites, we have previously reported that oligomeric proanthocyanidins (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, semi-synthetic, oleylated derivative.

Although estrogenic actions have been studied extensively for a number of polyphenols (see Kampa et al., 2007, for an extensive review), their 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 recent report further suggests also a potential membrane-initiated action of catechin analogs (Bastianetto et al., 2009), rapidly modifying PKC isoforms. Our previous results (Nifli et al., 2005) and data presented here suggest that indeed 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 as agonists of membrane androgen sites, an element which might be of a potential therapeutic importance.

The nature of membrane androgen sites has not been elucidated until now. Their characterization resides to 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 Kaarbo et al., 2007, for a recent review) and the transcriptional regulation of a number of genes, different from those initiated from unconjugated testosterone (Notas et al., 2010). There are evidences that they might be G-coupled protein receptors (Kaarbo 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, non-yet identified, proteins. Our data provide some new clues to this discussion. Indeed, we show that testosterone binding and actions on both AR-bearing (LnCaP) and/or AR-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 Figure 1, it becomes obvious that some specific molecular elements are needed for such an effect: (i) the beta configuration of the 4-8-link between the two flavanol moieties, (ii) the "2,3-cis" configuration (epicatechin 2*R*,3*R*-series) of the monomers (especially the alpha configuration of the hydroxyl group at position 3F) and (iii) 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 recently explored in the case of estrogen receptors, 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 and especially oleylated-B2 is not restricted to prostate cancer cells *in vitro*, but extended also *in vivo*, to BALBc$^{-/-}$ mice, xenografted with DU145 human prostate cancer cells. B2 and especially oleylated-B2 exerts a 50% inhibitory effect on tumor growth, in animals which 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 pro-apoptotic 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, while 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 and 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 (as 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.

ACKNOWLEDGEMENTS

The excellent technical assistance of Mrs Maria Klinaki is acknowledged.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: M. Kampa, EN. Stathopoulos, J. Vercauteren, E. Castanas

Conducted experiments: M. Kampa, K. Theodoropoulou, F. Mavromati, V. Pelekanou, G. Notas, ED. Lagoudaki, AP. Nifli, C. Morel-Salmi

Contributed new reagents or analytic tools: C. Morel-Salmi, J. Vercauteren

Performed data analysis: M. Kampa, E. Castanas

Wrote or contributed to the writing of the manuscript: M. Kampa, V. Pelekanou, G. Notas, AP. Nifli, C. Morel-Salmi, EN. Stathopoulos, J. Vercauteren, E. Castanas

Finally approved the manuscript: M. Kampa, V. Pelekanou, J. Vercauteren, E. Castanas

REFERENCES

Balas L, Vercauteren J and Laguerre M (1995) 2D NMR structure elucidation of proanthocyanidins: the special case of the catechin-(4 alpha-8)-catechin-(4 alpha-8)-catechin trimer. *Magn Reson Chem* **33**:85-94.

Bastianetto S, Dumont Y, Han Y and Quirion R (2009) Comparative neuroprotective properties of stilbene and catechin analogs: action via a plasma membrane receptor site? *CNS Neurosci Ther* **15**:76-83.

Bax A and Summers MF (1986) [1]H and [13]C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. *J Am Chem Soc* **108**:2093-2094.

Benten WP, Lieberherr M, Giese G, Wrehlke C, Stamm O, Sekeris CE, Mossmann H and Wunderlich F (1999) Functional testosterone receptors in plasma membranes of T cells. *FASEB J* **13**:123-133.

Butt MS and Sultan MT (2009) Green tea: nature's defense against malignancies. *Crit Rev Food Sci Nutr* **49**:463-473.

da Silva R, M. J, Rigaud J, Cheynier V, Cheminat A and Moutounet M (1991) Procyanidin dimers and trimers from grape seeds. *Phytochemistry* **30**:1259-1264.

Dambaki C, Kogia C, Kampa M, Darivianaki K, Nomikos M, Anezinis P, Theodoropoulos PA, Castanas E and Stathopoulos EN (2005) Membrane testosterone binding sites in prostate carcinoma as a potential new marker and therapeutic target: study in paraffin tissue sections. *BMC Cancer* **5**:148.

Delaunay J-C, Castagnino C, Cheze C and Vercauteren J (2002) Preparative isolation of polyphenolic compounds from Vitis vinifera by centrifugal partition chromatography. *J. Chrom. A* **964**:123-128.

Gu S, Papadopoulou N, Gehring EM, Nasir O, Dimas K, Bhavsar SK, Foller M, Alevizopoulos K, Lang F and Stournaras C (2009) Functional membrane androgen receptors in colon tumors trigger pro-apoptotic responses in vitro and reduce drastically tumor incidence in vivo. *Mol Cancer* **8**:114.

Hammes SR and Levin ER (2007) Extranuclear steroid receptors: nature and actions. *Endocr Rev* **28**:726-741.

Hatzoglou A, Kampa M, Kogia C, Charalampopoulos I, Theodoropoulos PA, Anezinis P, Dambaki C, Papakonstanti EA, Stathopoulos EN, Stournaras C, Gravanis A and Castanas E (2005) Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. *J Clin Endocrinol Metab* **90**:893-903.

Hatzoglou A, Romain S, Dalapascha H and Castanas E (1994) Acidification reveals a greater number of Epidermal Growth Factor Receptors in human membrane preparations. *Cli Chim Acta* **227**:97-109.

Kaarbo M, Klokk TI and Saatcioglu F (2007) Androgen signaling and its interactions with other signaling pathways in prostate cancer. *Bioessays* **29**:1227-1238.

Kampa M, Hatzoglou A, Notas G, Damianaki A, Bakogeorgou E, Gemetzi C, Kouroumalis E, Martin PM and Castanas E (2000) Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. *Nutr Cancer* **37**:223-233.

Kampa M, Kogia C, Theodoropoulos PA, Anezinis P, Charalampopoulos I, Papakonstanti EA, Stathopoulos EN, Hatzoglou A, Stournaras C, Gravanis A and Castanas E (2006)

Activation of membrane androgen receptors potentiates the antiproliferative effects of paclitaxel on human prostate cancer cells. *Mol Cancer Ther* **5**:1342-1351.

Kampa M, Nifli AP, Notas G and Castanas E (2007) Polyphenols and cancer cell growth. *Rev Physiol Biochem Pharmacol* **159**:79-113.

Kampa M, Papakonstanti EA, Hatzoglou A, Stathopoulos EN, Stournaras C and Castanas E (2002) The human prostate cancer cell line LNCaP bears functional membrane testosterone receptors that increase PSA secretion and modify actin cytoskeleton. *FASEB J* **16**:1429-1431.

Kampa M, Pelekanou V and Castanas E (2008) Membrane-initiated steroid action in breast and prostate cancer. *Steroids* **73**:953-960.

Kelly MJ and Levin ER (2001) Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* **12**:152-156.

Kennedy J and Jones G (2001) Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J. Agric. Food Chem.* **49**:1740-1746.

Kumar MV and Tindall DJ (1998) Transcriptional regulation of the steroid receptor genes. *Prog Nucleic Acid Res Mol Biol* **59**:289-306.

Levin ER (2009) Plasma membrane estrogen receptors. *Trends Endocrinol Metab* **20**:477-482.

Liao S (2001) The medicinal action of androgens and green tea epigallocatechin gallate. *Hong Kong Med J* **7**:369-374.

Manach C, Williamson G, Morand C, Scalbert A and Remesy C (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* **81**:230S-242S.

Marino M and Ascenzi P (2006) Steroid hormone rapid signaling: the pivotal role of S-palmitoylation. *IUBMB Life* **58**:716-719.

Nifli AP, Bosson-Kouame A, Papadopoulou N, Kogia C, Kampa M, Castagnino C, Stournaras C, Vercauteren J and Castanas E (2005) Monomeric and oligomeric flavanols are agonists of membrane androgen receptors. *Exp Cell Res* **309**:329-339.

Notas G, Pelekanou V, Castanas E and Kampa M (2010) Conjugated and non-conjugated androgens differentially modulate specific early gene transcription in breast cancer in a cell-specific manner. *Steroids* **75**:611-618.

Papakonstanti EA, Kampa M, Castanas E and Stournaras C (2003) A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. *Mol Endocrinol* **17**:870-881.

Pedram A, Razandi M, Sainson RC, Kim JK, Hughes CC and Levin ER (2007) A conserved mechanism for steroid receptor translocation to the plasma membrane. *J Biol Chem* **282**:22278-22288.

Pelekanou V, Kampa M, Kafousi M, Dambaki K, Darivianaki K, Vrekoussis T, Sanidas E, Tsiftsis DD, Stathopoulos EN and Castanas E (2007) Erythropoietin and its receptor in breast cancer: correlation with steroid receptors and outcome. *Cancer Epidemiol Biomarkers Prev* **16**:2016-2023.

Pelekanou V, Notas G, Sanidas E, Tsapis A, Castanas E and Kampa M (2010) Testosterone membrane-initiated action in breast cancer cells: Interaction with the androgen signaling pathway and EPOR. *Mol Oncol* **4**:135-149.

Ren F, Zhang S, Mitchell SH, Butler R and Young CY (2000) Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. *Oncogene* **19**:1924-1932.

Seyle H (1942) Correlations between the chemical structure and the pharmacological actions of the steroids. *Endocrinology* **30**:437-453.

Soini Y, Paakko P and Lehto VP (1998) Histopathological evaluation of apoptosis in cancer. *Am J Pathol* **153**:1041-1053.

Walker WH (2003) Nongenomic actions of androgen in Sertoli cells. *Curr Top Dev Biol* **56**:25-53.

Wang H, Yu D, Agrawal S and Zhang R (2003) Experimental therapy of human prostate cancer by inhibiting MDM2 expression with novel mixed-backbone antisense oligonucleotides: in vitro and in vivo activities and mechanisms. *Prostate* **54**:194-205.

Wang Z, Zhang X, Shen P, Loggie BW, Chang Y and Deuel TF (2005) Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. *Biochem Biophys Res Commun* **336**:1023-1027.

Wang Z, Zhang X, Shen P, Loggie BW, Chang Y and Deuel TF (2006) A variant of estrogen receptor-{alpha}, hER-{alpha}36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. *Proc Natl Acad Sci U S A* **103**:9063-9068.

Zhou HB, Chen JJ, Wang WX, Cai JT and Du Q (2005) Anticancer activity of resveratrol on implanted human primary gastric carcinoma cells in nude mice. *World J Gastroenterol* **11**:280-284.

Zhu Y, Rice CD, Pang Y, Pace M and Thomas P (2003) Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci U S A* **100**:2231-2236.

FOOTNOTES

Work partially supported by grants from the Greek Ministry of Health (ΚΕΣΥ, #128937/2008) and the Rersearch Account of the University of Crete.

VP Present affiliation: Laboratoire d'Anatomie Pathologique, Institut Jules Bordet-Centre des Tumeurs de l' ULB, Rue Héger-Bordet 1, 1000 Brussels, Belgium, e-mail: vasiliki.pelekanou@ulb.ac.be

APN Present affiliation: School of Medicine, University of Thessaly, Greece, e-mail: f_nifli@hotmail.com

KT and FM have equally contributed to this work

Conflict of interest: MK, KT, FM, VP, APN, JV and EC are inventors in patent applications related to the subject.

LEGENDS FOR FIGURES

Figure 1

Chemical structures of the substances used in the study

Figure 2

Effect of OPCs B1-B4 (**A**), galloylated derivatives (**B**) and dioleylated B2 (**C**) on growth of androgen-sensitive (LnCaP) and resistant (DU145) human prostate cancer cells. Figure presents normalized results as compared to control (non-treated) cells. The effect was measured after two cell cycles. Mean±SEM of three different experiments, performed in triplicate.

Figure 3

A: Displacement of [^{3}H]testosterone from membrane binding sites of DU145 cell membranes by B2, B3 and their oleylated conjugates. Figure presents means±SEM 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.

Figure 4

In vivo effects of oleylated B2, in the reduction of BALBc$^{-/-}$ DU145 tumors

Ten-week male BALBc$^{-/-}$ animals were injected with 5x10^{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 Tumor volume (**A**) and inhibitory rate (**B**) are calculated as described in Material and Methods. In **C**, 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.

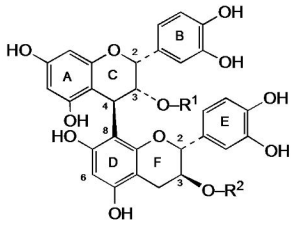
TABLES

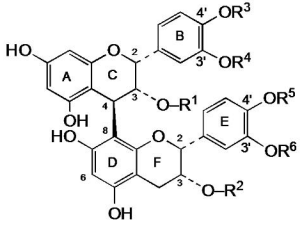
Table 1

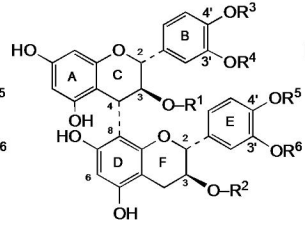
Maximal inhibition and IC_{50}s of substances used in the study on cell proliferation of prostate cancer cell lines

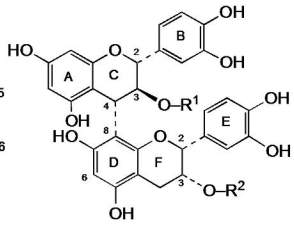
	LnCaP		DU-145	
	Max Inhibition	IC_{50} (nM)	Max Inhibition	IC_{50} (nM)
Testo-BSA	0.74±0.06	0.7±0.05	0.62±0.08	0.8±0.07
B1	0.93±0.06	-	0.88±0.03	74.3±0.06
B2	0.9±0.02	30.4±3.54	0.87±0.06	0.6±0.02
B3	0.87±0.04	0.5±0.01	0.79±0.11	47.4±6.87
B4	0.88±0.03	0.5±0.04	0.85±0.04	48.1±4.88
Epicatechin	0.40±0.03	0.5±0.07	0.58±0.03	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



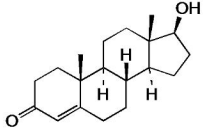




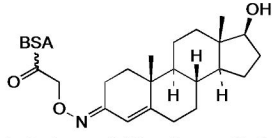


R^1= H, R^2= H	procyanidin **B1** (–)-epicatechin-(4β-8)-(+)-catechin	procyanidin **B2** (–)-epicatechin-(4β-8)-(–)-epicatechin	procyanidin **B3** (+)-catechin-(4α-8)-(+)-catechin	procyanidin **B4** (+)-catechin-(4α-8)-(–)-epicatechin
R^1= H, R^2= G	**B1** 3F-*O*-gallate	**B2** 3F-*O*-gallate		
R^1= G, R^2= G		**B2** 3C,3F-di-*O*-gallate		

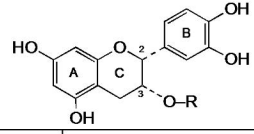
R^3	R^4		R^5	R^6		
	H	and		H	3' or 4'B, 3' or 4'E-di-*O*-oleyl-procyanidin **B2**	3' or 4'B, 3' or 4'E-di-*O*-oleyl-procyanidin **B3**
H			H			



testosterone



testosterone-3-(*O*-carboxymethyl)-oxime-BSA (testosterone-BSA)



R= H	(–)-epicatechin
R= G	3-*O*-galloyl-(–)-epicatechin

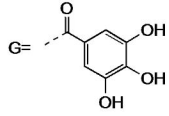


Figure 1

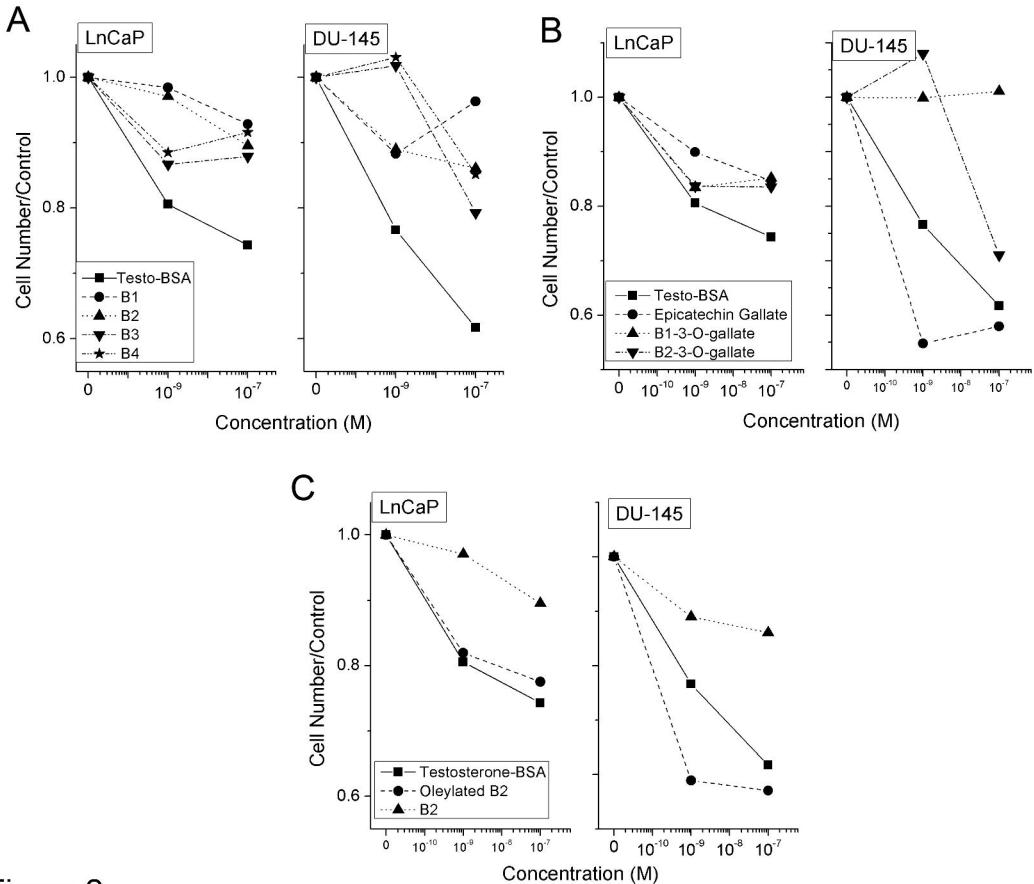
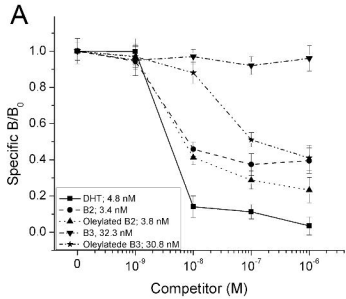

A
LnCaP
DU-145
Cell Number/Control
1.0
0.8
0.6
Testo-BSA
B1
B2
B3
B4
0
10^{-9}
10^{-7}
Concentration (M)
B
LnCaP
DU-145
Cell Number/Control
1.0
0.8
0.6
Testo-BSA
Epicatechin Gallate
B1-3-O-gallate
B2-3-O-gallate
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
Concentration (M)
C
LnCaP
DU-145
Cell Number/Control
1.0
0.8
0.6
Testosterone-BSA
Oleylated B2
B2
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
Concentration (M)

Figure 2


A
Specific B/B0
1.0
0.8
0.6
0.4
0.2
0.0
DHT; 4.8 nM
B2; 3.4 nM
Oleylated B2; 3.8 nM
B3, 32.3 nM
Oleylatede B3; 30.8 nM
0
10-9
10-8
10-7
10-6
Competitor (M)

B

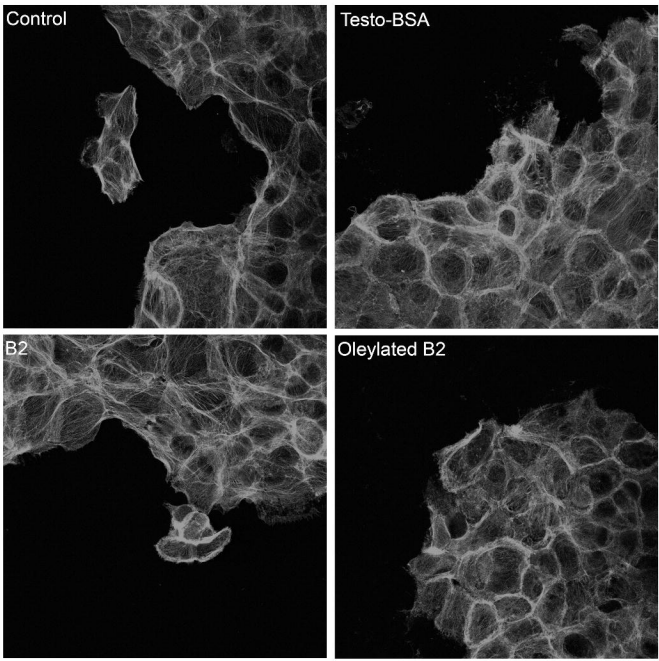

Control
Testo-BSA
B2
Oleylated B2

Figure 3

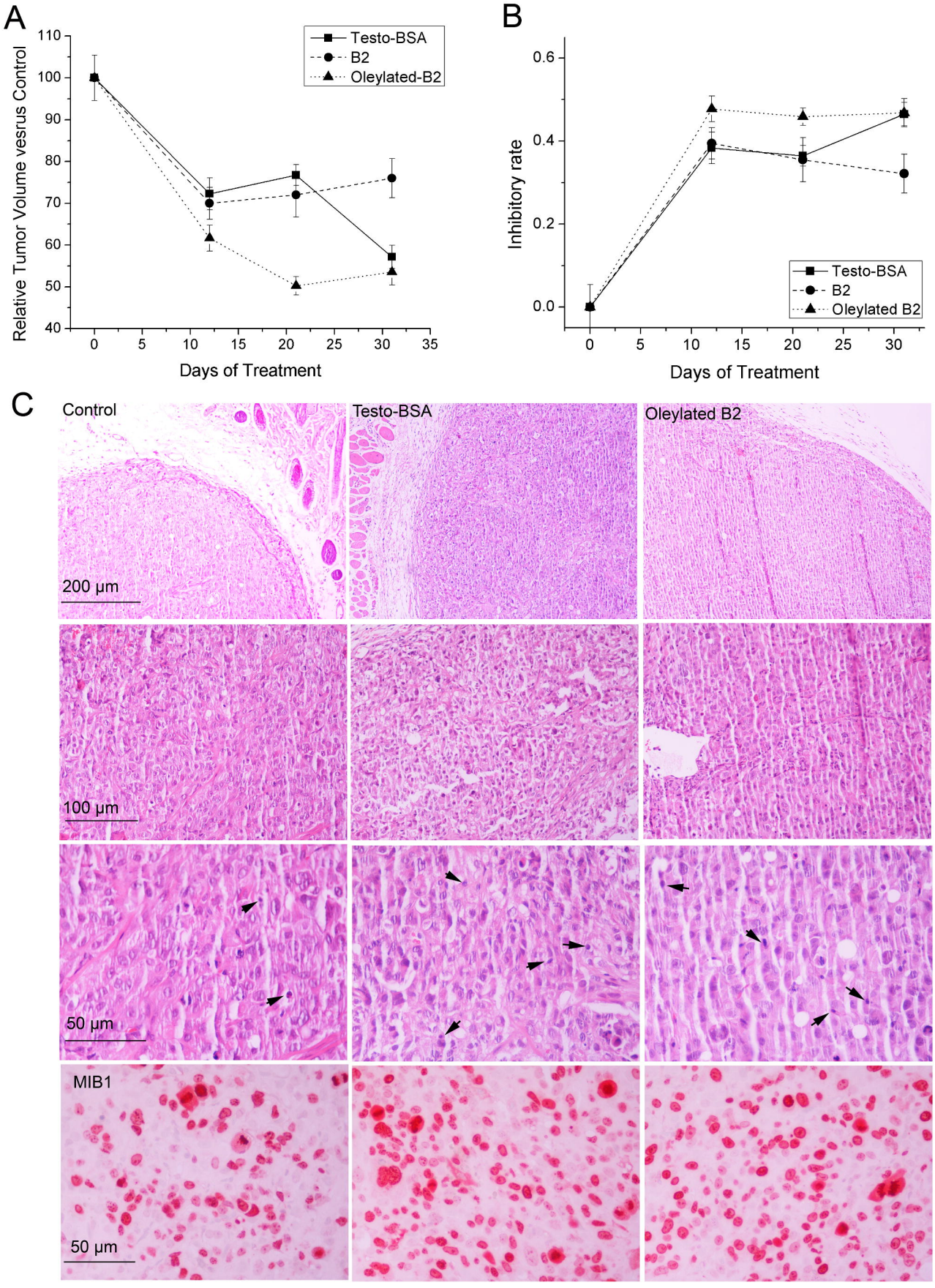

A
Relative Tumor Volume vesrus Control
Days of Treatment
Testo-BSA
B2
Oleylated-B2
B
Inhibitory rate
Days of Treatment
Testo-BSA
B2
Oleylated B2
C
Control
Testo-BSA
Oleylated B2
200 μm
100 μm
50 μm
MIB1
50 μm

Figure 4