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

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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°C in a humidified atmosphere of 5% CO₂ 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 épicatechine 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
(Rt = 11.5 min), procyanidin B1 (Rt= 12.0 min), procyanidin B4 (Rt= 14.6 min), catechin (Rt= 15.5 min),
procyanidin B2 (Rt = 17.1 min), procyanidin B1 3F-O-gallate (Rt= 18.3 min),
procyanidin B2 3F-O-gallate (Rt= 19.4 min), epicatechin (Rt= 21.2 min), trimer 3-O-gallate (Rt= 22.3 min),
epicatechin-3-O-gallate (Rt= 25.5 min), procyanidin B2 3C,3F-di-O-gallate (Rt= 33.0 min). These proanthocyanidins were already isolated and identified in grapes (da Silva et al.,
Preparation of oleylated B2 and B3 derivative

To a solution of dimer B2 or B3 (2 g; 3.5 mmol) in 300 ml CHCl3, was added 766 ml 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 CHCl3 was added dropwise over a 2 hours period. The CHCl3 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 NH4Cl was added, prior the extraction by CHCl3. 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 [3H]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°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°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 $5 \times 10^6$ 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), 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):

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

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-conjugated testosterone, testosterone-BSA) as revealed by flow cytometry. In addition, $[^3H]$-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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 IC50s. 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 IC50 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 2R,3R-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.
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AUTHORSHIP CONTRIBUTIONS

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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


FOOTNOTES

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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 [3H]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^-7M 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.
### Table 1

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

<table>
<thead>
<tr>
<th>Substance</th>
<th>LnCaP Inhibition</th>
<th>LnCaP IC$_{50}$ (nM)</th>
<th>DU-145 Inhibition</th>
<th>DU-145 IC$_{50}$ (nM)</th>
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<tbody>
<tr>
<td>Testo-BSA</td>
<td>0.74±0.06</td>
<td>0.7±0.05</td>
<td>0.62±0.08</td>
<td>0.8±0.07</td>
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<tr>
<td>B1</td>
<td>0.93±0.06</td>
<td>-</td>
<td>0.88±0.03</td>
<td>74.3±0.06</td>
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<tr>
<td>B2</td>
<td>0.9±0.02</td>
<td>30.4±3.54</td>
<td>0.87±0.06</td>
<td>0.6±0.02</td>
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<tr>
<td>B3</td>
<td>0.87±0.04</td>
<td>0.5±0.01</td>
<td>0.79±0.11</td>
<td>47.4±6.87</td>
</tr>
<tr>
<td>B4</td>
<td>0.88±0.03</td>
<td>0.5±0.04</td>
<td>0.85±0.04</td>
<td>48.1±4.88</td>
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<tr>
<td>Epicatechin</td>
<td>0.40±0.03</td>
<td>0.5±0.07</td>
<td>0.58±0.03</td>
<td>0.5±0.04</td>
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<tr>
<td>Epicatechin-3-O-gallate</td>
<td>0.86±0.04</td>
<td>0.7±0.03</td>
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<td>B1-3F-O-gallate</td>
<td>0.83±0.02</td>
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<td>B2-3F-O-gallate</td>
<td>0.84±0.02</td>
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<td>0.71±0.07</td>
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<tr>
<td></td>
<td>B2</td>
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<td>B3</td>
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<tr>
<td>Oleylated</td>
<td>0.78±0.05</td>
<td>0.6±0.03</td>
<td>0.57±0.05</td>
<td>0.4±0.03</td>
</tr>
<tr>
<td>Oleylated</td>
<td>0.79±0.06</td>
<td>0.65±0.04</td>
<td>0.74±0.03</td>
<td>35.4±6.21</td>
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</table>
| $R^1=H$, $R^2=H$ | procyanidin B1 | \((-\text{-epicatechin-}(4\beta-8)-\text{(+)-catechin})$ | procyanidin B2 | \((-\text{-epicatechin-}(4\beta-8)-\text{(-)-epicatechin})$ | procyanidin B3 | \((+\text{-catechin-}(4\alpha-8)-\text{(+)-catechin})$ | procyanidin B4 | \((+\text{-catechin-}(4\alpha-8)-\text{(-)-epicatechin})$
<table>
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<tbody>
<tr>
<td>$R^1=H$, $R^2=G$</td>
<td>B1 3F-O-gallate</td>
<td>B2 3F-O-gallate</td>
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<tr>
<td>$R^1=G$, $R^2=G$</td>
<td>B2 3C,3F-di-O-gallate</td>
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<table>
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<tr>
<th>$R^3$</th>
<th>$R^4$</th>
<th>$R^5$ and $R^6$</th>
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<td>(\text{C}<em>1H</em>{33})</td>
<td>H</td>
<td>(\text{C}<em>1H</em>{33}) and H</td>
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<tr>
<td>(\text{C}<em>1H</em>{33})</td>
<td>O</td>
<td>(\text{C}<em>1H</em>{33}) and O</td>
</tr>
<tr>
<td>H</td>
<td>O</td>
<td>H</td>
</tr>
</tbody>
</table>

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

![Testosterone](image)

![Testosterone-3-(O-carboxymethyl)-oxime-BSA](image)

![R=H](image) \((-\text{-epicatechin})$

![R=G](image) 3-O-galloyl-\((-\text{-epicatechin})$
Figure 2
Figure 3

(A) Graph showing the specific binding of various competitors at different concentrations. The x-axis represents the competitor concentration (M), and the y-axis represents the specific binding (BB0). Different competitors are indicated by different symbols and concentrations.

(B) Images showing control cells and cells treated with Testo-BSA, B2, and Oleylated B2. The images illustrate the effects of these treatments on cell morphology.
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, Artemisia-Phoebe Nifli, Cécile Morel-Salmi, Efstathios N. Stathopoulos, Joseph Vercauteren, Elias Castanas

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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 saturation binding, as described in the Material and Methods section. Inserts present analysis of saturation 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.
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).