Estrogen receptor β and the progression of prostate cancer: role of 5α-androstane-3β,17β-diol

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Abstract

Prostate cancer (PC) develops in response to an abnormal activation of androgen receptor induced by circulating androgens and, in its initial stages, is pharmacologically controlled by androgen blockade. However, androgen ablation therapy often allows androgen-independent PC development, generally characterized by increased invasiveness. We previously reported that 5α-androstane-3β,17β-diol (3β-Adiol) inhibits the migration of PC cell lines via the estrogen receptor β (ERβ) activation. Here, by combining in vitro assays and in vivo imaging approaches, we analyzed the effects of 3β-Adiol on PC proliferation, migration, invasiveness, and metastasis in cultured cells and in xenografts using luciferase-labeled PC3 (PC3-Luc) cells. We found that 3β-Adiol not only inhibits PC3-Luc cell migratory properties, but also induces a broader anti-tumor phenotype by decreasing the proliferation rate, increasing cell adhesion, and reducing invasive capabilities in vitro. All these 3β-Adiol activities are mediated by ERβ and cannot be reproduced by the physiological estrogen, 17β-estradiol, suggesting the existence of different pathways activated by the two ERβ ligands in PC3-Luc cells. In vivo, continuous administration of 3β-Adiol reduces growth of established tumors and counteracts metastasis formation when PC3-Luc cells are engrafted s.c. in nude mice or are orthotopically injected into the prostate. Since 3β-Adiol has no androgenic activity, and cannot be converted to androgenic compounds, the effects here described entail a novel potential application of this agent against human PC.

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Introduction

Prostate cancer (PC) is a hormone-dependent malignancy, which is frequently present in aging males (Matsushima et al. 1999) when a deregulated local production of androgens and an abnormal androgen receptor (AR) activation may promote an uncontrolled cell proliferation and, eventually, carcinogenesis. In the early stages, PC growth may be therapeutically controlled by androgen blockade (Cunha et al. 1987), but at later stages, the pathology (Balk 2002) may become refractory as a consequence of clonal selection of androgen-independent ‘foci’ (Stanbrough et al. 2006). Androgen-resistant tumors are generally characterized by an increased invasiveness. In prostate cells, the major male sex hormone testosterone may directly activate AR, or be irreversibly reduced to dihydrotestosterone (DHT), by the 5α-reductase enzyme (Pozzi et al. 2003, Poletti et al. 2005). DHT is much more potent than testosterone as an AR activator (Wilson et al. 1993). The 5α-reductase not only amplifies the androgenic signal (Normington & Russell 1992, Wilson et al. 1993, Poletti et al. 2001, 2005), but also prevents estrogen formation by subtracting testosterone from the process of aromatization (Roselli & Resko 1993, Poletti et al. 1997, 2001).
In previous studies, we have demonstrated that 3β-Adiol acting through the activation of ERβ (Weihua et al. 2001, 2002a,b). In the gland, DHT may be either reversibly 3α- or irreversibly 3β-hydroxylated by the different 3α- and 3β-hydroxysteroid dehydrogenases respectively (Steckelbroeck et al. 2004); these transformations generate two metabolites respectively 3α-diol and 3β-Adiol, which are both unable to bind the AR. Instead, 3β-Adiol displays a high affinity for ERβ (Kuiper et al. 1998, Nilsson et al. 2001), and it has been proposed that this metabolite may play a key role in prostate development (Warner & Gustafsson 1995, Weihua et al. 2001, 2002a,b, Gustafsson 2003). Moreover, 3β-Adiol and 17β-estradiol (E2) differentially activate ERβ signaling in PC cells (Guerini et al. 2005), suggesting that 3β-Adiol could act as an endogenous selective ER modulator (SERM) in male prostate. ERβ signaling, in contrast to ERα, seems to act as a suppressor of prostate growth, and may be positively involved in breast cancer (Lazennec et al. 2001, Cheng et al. 2004). In previous studies, we have demonstrated that 3β-Adiol, acting through the activation of ERβ, inhibits the migratory capabilities of AR-positive and AR-negative prostate cell lines in vitro (Guerini et al. 2005). Other studies suggested that using the same ER pathway, 3β-Adiol counteracts PC cell proliferation in vitro (Weihua et al. 2001, 2002a, Imamov et al. 2004, Koehler et al. 2005). These data as a whole indicate that testosterone might control prostate physiology through several contrasting mechanisms: a) androgenic signals via AR/testosterone and AR/DHT complexes, b) estrogenic signals via ER (α and β)/E2 and c) alternative estrogenic signaling via ER (β)/3β-Adiol.

The goal of this study was to assess the effects of 3β-Adiol on PC cell proliferation, migration, and invasiveness in vitro as well as in vivo in xenograft models of tumorigenesis in nude mice.

Materials and methods

Reagents

3β-Adiol, DHT, and E2 (R, R′)-cis-diethyltetrahydro-2,8-chrysenediol (R, R′-THC) were obtained from Sigma–Aldrich. 2,2,4,6,6-17α,21,21,21-D9-PROG (D9-PROG) was obtained from Medical Isotopes (Pelham, NH, USA). ICI 182,780 was kindly donated by AstraZeneca. Matrigel was obtained from Becton Dickinson Biosciences Clontech. Solid phase extraction (SPE) cartridges (Discovery DS-C18 500 mg) were obtained from Supelco, Milan, Italy. All solvents and reagents were HPLC grade (Sigma–Aldrich). For the experiments in vivo, 3β-Adiol was dissolved in sesame oil, and injected s.c. at a dose of 2.5 mg/kg. Control animals were injected with the same amount of vehicle.

Cell culture

The cell lines DU145 and PC3 were originally obtained from American Type Culture Collection (Rockville, MD, USA). The luciferase-labeled PC3 (PC3-Luc) cells (Buijs et al. 2007) were kindly provided from Prof. C Lowik (LUMC, Leiden, The Netherlands). Cells were routinely grown in RPMI 1640 medium (Biochrom KG, Berlin, Germany), supplemented with 5% fetal bovine serum (FBS) that was obtained from Gibco BRL, glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO2:95% air at 37 °C.

Cell growth studies

DU145, PC3 cells, and PC3-Luc cells, plated in 10 mm dishes in serum-free medium, were treated for 48 h with graded doses of 3β-Adiol (1.0–10 nM). Cells were then harvested and counted by using a hemocytometer. In another series of experiments, 1 μCi/ml [3H]thymidine was added to PC3-Luc cells. PC3-Luc cells, cultured in serum-free medium, were incubated for 24 h with 3β-Adiol (1.0–10 nM) or E2 (1.0–10 nM). Following [3H]thymidine addition after 6 h, cells were washed in PBS, and radioactivity was counted in β-counter 1600CA TRI-CARB Perkin-Elmer (Monza, Italy). To test the specificity of the effects of the DHT metabolite on cell proliferation and [3H]thymidine incorporation, we conducted, in PC3-Luc cells, a 48-h treatment with 3β-Adiol (10 nM) either alone or in combination with either the ICI 182,780 (1 μM) or the R, R′-THC (1 μM). ERβ silencing was performed using an shRNA already proven to be active against ERβ mRNA expression in PC cells (Arnold et al. 2007, Treeck et al. 2010). shRNA transfection was performed as previously described (Guerini et al. 2005, Marron et al. 2005). Briefly, PC3-Luc cells (500 000 cells/well) were plated in a 6-well dish (cells were seeded in RPMI containing 5% FBS). The next day, the medium was replaced with 2 ml fresh culture medium. Transfection was performed using 4 μl of transferrin solution (3.2 μg/μl, Sigma) plus 3 μl of Lipofectamine (Invitrogen) and 1 μg of the shRNA plasmid mix (0.25 μg of each shRNA composing the mix, according to (Arnold et al. 2007, Treeck et al. 2010)) or control scramble shRNA in RPMI, and added to the cultured cells. In all experiments involving steroid hormone treatments,
FBS was replaced with charcoal-stripped FBS (CS-FBS; 5%) to eliminate endogenous steroids. For transfection with ERβ shRNA, a mixture of four plasmids (SureSilencing, SABiosciences, DBA, Milano, Italy) was used containing the ERβ-specific shRNA insert sequences: a) 5'-cgccattacactctgtat-3’ (ESR2 exon 1), b) 5'-tccagcagtcactaact-3’ (ESR2 exon 1), c) 5'-aggccatgatcctgctcaatt-3’ (ESR2 exon 6), and d) 5’-gatcttgtcaactccagat-3’ (ESR2 exon 6/7) (Arnold et al. 2007, Treeck et al. 2010). As a negative control, the same plasmid with the unspecific insert 5'-ggaatctcattcgatgcatac-3' was used.

The results of three separate experiments are presented as the mean ± S.D. Each experimental group was composed of eight replicates.

**Adhesion assay**

Forty-eight-well flat-bottomed plastic plates were coated with 20 µg/ml laminin (Chemicon International, Milan, Italy) or 20 µg/ml fibronectin (Sigma). PC3-Luc cells, pre-treated for 48 h with 3β-Adiol (10 nM) either alone or in combination with either the ICI 182,780 (1 µM) or the R,R'-THC (1 µM), were collected and plated at 200,000 cells per well. ERβ silencing was performed as described in the previous section. Cells were allowed to adhere for 30 min at 37 °C. At the end of incubation, the cells were fixed in methanol, then stained with Diffquik (Biomap, Milan, Italy), and measured by absorption at 595 nm.

**Microchemotaxis assay**

Briefly, cell migration assay was performed using a 48-well Boyden chamber (Neuroprobe, Inc., Gaithersburg, MD, USA) containing 8 µm polycarbonate filters (Nucleopore, Concorezzo, Milan, Italy). Filters were coated on one side with 50 µg/ml laminin, rinsed once with PBS, and then placed in contact with the lower chamber containing RPMI 1640 medium. PC3-Luc cells, pre-treated for 48 h with 3β-Adiol (1.0–10 nM) and E2 (1.0–10 nM) either alone or in combination with ICI 182,780 (1 µM), were collected and then added in aliquots (75,000 cells/50 µl) to the top of each chamber and allowed to migrate through coated filters for 4 h. At the end of the incubation, the migrated cells attached on the lower membrane surfaces were fixed, stained with Diffquik (Biomap), and counted at a 40× magnification in standard optical microscopy.

The results of three separate experiments of migration are presented as the mean ± S.D. Each experimental group consists of 12 samples. The results are expressed as a percentage of migrated cells versus control cells.

**Matrigel gel assay**

PC3-Luc cells were collected and suspended in 2 µl Matrigel drops (800,000 cells) in a 3 cm (diameter) culture dish for 30 min at room temperature. Cell aggregates were covered with 500 µl of RPMI supplemented with 5% CS-FBS, and transferred to the cell culture incubator. The aggregates were treated with vehicle (0.01% ethanol) or 3β-Adiol (10 nM) for 48 h and observed daily under a light microscope; at the end of the treatment, cell aggregates were fixed and stained with Diffquik (Biomap), and phase-contrast images of the aggregates were taken.

**Experimental animals and pharmacological manipulations**

Six-to-eight-week-old BALB/c nu/nu male mice (Charles River, Calco, Italy) were maintained at the Animal Resource Facility at University of Milan (Italy). Experiments performed in this study were conducted according to the Guidelines for Care and Use of Experimental Animals. The use of experimental animal was approved by the Italian Ministry of Research and University, and was controlled by the panel of experts of the Department of Pharmacological Sciences at the University of Milan.

In orthotopic xenograft experiments, mice were daily injected s.c. with 2.5 mg/kg 3β-Adiol in sesame oil or with corresponding amount of vehicle (sesame oil).

Silastic pellets (Degania Silicone Ltd, Degania Bet, Israel) were prepared by cutting a silastic pipe (diameter 1.5 mm) into small segments of 1 cm long. These segments were filled with 3β-Adiol powder (or left empty in vehicle-treated mice), and closed to the edge with a double knot made by a surgical silk thread. Silastic pellets were then implanted s.c. on the left side of the mouse just behind the anterior leg using a trochar.

**Bioluminescence reporter imaging**

Mice were visualized with a Night Owl imaging unit (Berthold Technologies, Bad Wildbad, Germany) consisting of a Peltier-cooled charge-coupled device slow-scan camera equipped with a 25 mm f/0.95 lens. Images were generated by a Night Owl LB981 image processor, and transferred via video cable to a peripheral component interconnect frame grabber using WinLight32 software (Berthold Technologies). For the detection of bioluminescence, mice were anesthetized using an s.c. injection of 50 µl of ketamine–xylazine solution composed of 78% ketamine (Ketavet 50; Intervet, Peschiera Borromeo, Italy), 15% xylazine (2% solution, Rompun; Bayer),...
and 7% water. Mice then received an i.p. injection of 50 mg/kg D-luciferin (Promega) 20 min before bioluminescence quantification to obtain a uniform biodistribution of the substrate. We administered the substrate D-luciferin according to a procedure described previously (Ciana et al. 2003, 2005). Mice were placed in the light-tight chamber, and a grayscale photo of the animals was first taken with dimmed light. Photon emission was then integrated over a period of 5 min. Merging of the images enabled to visualize the body areas where photon emission occurred (luciferase signal was transformed in pseudocolors: blue, low; white, high). Quantification of photon emissions from tumor areas was done using WinLight32 imaging software (Berthold Technologies).

**Xenograft prostate tumor models**

In a first series of experiments, PC3-Luc cells (1×10⁶ and 5×10⁶) were s.c. injected in the shoulder of BALB/c nu/nu male nude mice, while for orthotopical models, 1×10⁵ and 1×10⁶ PC3-Luc cells were injected into the male prostate by surgical intervention on anesthetized animals. All mice were subsequently subjected to bioluminescence imaging (BLI) analysis as described above.

**Liquid chromatography-tandem mass spectrometry analysis**

Positive atmospheric pressure chemical ionization (APCI+) experiments were performed with a linear ion trap mass spectrometer (LTQ, ThermoElectron Co., San Jose, CA, USA) using nitrogen as sheath, auxiliary, and sweep gas. The instrument was equipped with a Surveyor liquid chromatography (LC) Pump Plus and a Surveyor Autosampler Plus (ThermoElectron Co). The mass spectrometer was employed in tandem mass spectrometry mode using helium as a collision gas.

The LC mobile phases were (A) H₂O/0.1% formic acid and (B) methanol (MeOH)/0.1% formic acid. The gradient (flow rate 0.5 ml/min) was as follows: T0, 70% A; T1.5, 70% A; T2, 55% A; T3, 55% A; T35, 36% A; T40, 25% A; T41, 1% A; T45, 1% A; T45.2, 70% A; and T55, 70% A. The split valve was set at 0–6.99 min to waste, 6.99–43.93 min to source, and 43.93–55 to waste. The Hypersil Gold column (100×3 mm, 3 μm; ThermoElectron Co.) was maintained at 40 °C. The injection volume was 25 μl, and the injector needle was washed with MeOH/water 1/1 (v/v). Peaks of the LC–MS/MS were evaluated using a Dell workstation by means of the software Excalibur release 2.0 SR2 (ThermoElectron Co). Samples were

![Figure 1](https://www.endocrinology-journals.org/734)

Figure 1 3β-Adiol and 17β-estradiol regulation of PC cell growth. Cell proliferation of DU145 (A), PC3 (B), and PC3-Luc (C) cells incubated for 48 h with increased concentrations of 3β-Adiol (0.1–10 nM). (D) [³H]thymidine incorporation on PC3-Luc cells incubated for 48 h with increasing concentrations of 3β-Adiol (0.1–10 nM) and 17β-estradiol (0.1–10 nM). Values (cell number or [³H]thymidine incorporation) are expressed as the percentage of hormone-treated versus vehicle-treated samples. The results of the experiments are presented as the mean ± s.d. Each experimental group was composed of eight replicates. Statistical analysis was performed by one-way ANOVA followed by Dunnett’s multiple comparison tests. *P<0.05 versus vehicle.
analyzed using the following transitions: m/z 324→100 for D9-PROG, m/z 291→255 for DHT, and m/z 257→(121+135+147+161+175+201) for 3β-Adiol.

Plasma samples were extracted and purified as previously described (Caruso et al. 2008, Pesaresi et al. 2010). Briefly, plasma samples (100–200 μl) were added with internal standard and with 2 ml of MeOH/acetic acid (99:1 v/v). After an overnight extraction at 4°C, samples were centrifuged at 15300 g for 5 min, and the pellet was extracted twice with 1 ml of MeOH/acetic acid (99:1 v/v). The organic phases were combined and dried with a gentle stream of nitrogen in a 40°C water bath. The samples were resuspended with 3 ml of MeOH/H2O (10:90 v/v) and passed through a SPE cartridge, previously activated with MeOH (5 ml) and MeOH:H2O (1:9 v/v; 5 ml); the steroids were eluted in MeOH, concentrated, and transferred in autosampler vials before the LC–MS/MS analysis.

DHT and 3β-Adiol were identified on the basis of both the retention time and the MS/MS spectrum of reference compounds. Quantitative analysis was performed on the basis of calibration curves prepared and analyzed using deuterated internal standard. Calibration curves were extracted and analyzed as described above for samples.

Statistical analysis

Statistical analysis, when necessary, was performed by one-way ANOVA followed by Dunnett’s or Tukey’s multiple comparison tests. P < 0.05 was considered statistically significant. For xenograft data analysis of treatment, group versus control was carried out with ANOVA followed by Student’s t-test.

For LC–MS/MS analysis, the linearity of the standard curve (r²), the accuracy (%), and the precision (CV%) inter-series were judged by GraphPad4 PRISM (version 4).

Results

3β-Adiol regulation of PC cell growth

In the initial set of experiments, we have measured the effects of increasing concentration of 3β-Adiol (0.1–10 nM) on AR-negative PC cell proliferation. In growth course experiments, 3β-Adiol significantly reduced (about 50% reduction at 10 nM, the maximal concentration tested), after 48-h treatment, the number of DU145, PC3, and PC3-Luc cells (Fig. 1A–C); the cell number reduction was due to inhibition of the proliferative rate of the cells as confirmed by thymidine incorporation assays (Fig. 1D). Since our previous results demonstrated that the anti-migratory effect of 3β-Adiol on PC cells was not mediated by the AR, but by the ERβ isoform (Guerini et al. 2005), we investigated the anti-proliferative activity of 3β-Adiol in the presence of the pure anti-estrogen ICI 182,780 (1 μM) on the PC3-Luc cell line. Pretreatment of the cells with ICI 182,780 prevented the inhibitory effects of 3β-Adiol in both growth course and thymidine incorporation experiments (Fig. 2). Very similar data have been obtained using an anti-ERβ-selective antagonist (ERz agonist) such as the R,R'-THC at doses of 1 μM. (Supplementary Figure 1A, see section on supplementary data given at the end of this article); in fact, also R,R'-THC was able to abolish the anti-proliferative activity of 3β-Adiol on PC3-Luc cells. Moreover, the anti-proliferative effects of 3β-Adiol on
PC3-Luc cells were completely counteracted by silencing ERβ expression with a specific shRNA against this receptor (Supplementary Figure 1B; Arnold et al. 2007, Treeck et al. 2010), proving that ERβ is the molecular mediator of 3β-Adiol action. Therefore, considering that the only ER isoform expressed in DU145, PC3, and PC3-Luc cells is ERβ, as demonstrated by real-time PCR and western blot experiments (Supplementary Figure 2, see section on supplementary data given at the end of this article; Guerini et al. 2005), we concluded that the anti-proliferative activity of 3β-Adiol is mediated by this receptor. In addition, similar to what we reported for cell migration (Guerini et al. 2005), this activity was not shared by the physiological ER ligand E2 (Fig. 1D).

3β-Adiol regulation of PC3-Luc cell adhesion

Next, we analyzed the effects of 3β-Adiol on cell adhesion on laminin or fibronectin matrix substrates to mimic different extracellular adhesion conditions. To this aim, we pre-treated PC3-Luc cells with 3β-Adiol (10 nM) for 48 h either alone or in combination with ICI 182,780 (1 μM); cells were then seeded on laminin- or fibronectin-coated plates, and the adherent versus floating cell ratios were evaluated 30 min after plating. The results obtained demonstrated that 3β-Adiol significantly increased the capability of PC3-Luc cells to adhere to both laminin-coated (Fig. 3A) and fibronectin-coated (Fig. 3B) plates. The effect of 3β-Adiol on the adhesion properties of PC3-Luc cells was analyzed in the presence of the pure ER antagonist ICI 182,780 (Fig. 3) or anti-ERβ-selective antagonist R,R'-THC (1 μM; Supplementary Figure 3A, see section on supplementary data given at the end of this article). In fact, both antagonists (pure or ERβ-selective) significantly counteracted the pro-adhesive properties of 3β-Adiol on PC3-Luc cells estimated using the adhesion assay. Moreover, the pro-adhesive effects of 3β-Adiol on PC3-Luc cells were completely counteracted by silencing ERβ expression with shRNA (Supplementary Figure 3B; Arnold et al. 2007, Treeck et al. 2010), supporting the previous data which demonstrated that ERβ is the molecular mediator of 3β-Adiol action. Therefore, the pro-adhesion activity of 3β-Adiol is mediated by ERβ activation.

Effects of 3β-Adiol on PC3-Luc cell migration and invasion

Given that detachment from extracellular matrix is required for cell migration, we tested whether the pro-adhesive effects of 3β-Adiol could be correlated with reduced migratory properties of PC3-Luc cells using a haptotaxis assay previously described (Guerini et al. 2005). Haptotaxis was evaluated in a Boyden’s chamber using laminin-coated membranes, because preliminary experiments had indicated that PC3-Luc cells fail to migrate in the absence of a specific substrate (not shown). PC3-Luc cells pre-treated for 48 h with 3β-Adiol were characterized by a strong decrease of cell migration on laminin when compared to untreated cells (Fig. 4A). A significant effect was measured already at the lowest concentration tested; in particular, the exposure to 1 and 10 nM 3β-Adiol reduced the migratory activity of PC3-Luc respectively of 43 and 57%. On the contrary, E2 (1–10 nM), in the same experimental conditions, did not modify the migratory capabilities of PC3-Luc cells (Fig. 4B). The inhibitory effect of 3β-Adiol on the motility of PC3-Luc cells was totally abolished by the presence of the ER antagonist ICI 182,780 (Fig. 4C) and, as already previously shown, also using the anti-ERβ-selective antagonist R,R'-THC (Guerini et al. 2005); this also
Effects of 3β-Adiol on PC3-Luc cell growth in xenografted mice

Altogether our in vitro results indicated that 3β-Adiol decreases significantly cell growth, adhesion, migration, and invasion in PC3-Luc cells. Thus, we investigated whether these effects could influence PC growth and metastasis in vivo using a BLI assay. To establish the parameter for evaluating growth inhibition, we have initially s.c. implanted 5x10^6 PC3-Luc cells in the shoulder of BALB/c nu/nu nude mice; tumor growth could be followed by BLI (Stell et al. 2007) and metastasis appeared at week 4 (Supplementary Figure 4, see section on supplementary data given at the end of this article). After these preliminary experiments, we decided to s.c. implant 5x10^6 PC3-Luc cells in one shoulder of 20 animals, and after 3 weeks, the tumors were clearly detectable by BLI (Fig. 5, begin of treatment). The animals were divided into two groups of ten mice per group displaying similar average photon emissions; one group was implanted with a silastic pellet continuously releasing 3β-Adiol, while the other group was implanted with an empty pellet. After 2 weeks of treatment, we performed a second BLI acquisition. The results showed that in the vehicle group, photon emission was increased at an average of 5.0 times, while in the 3β-Adiol group, photon emissions were only slightly increased (Fig. 5). From these experiments, we concluded that 3β-Adiol significantly inhibited proliferation of established tumors in nude mice. After that, we decided to measure the ability of the testosterone metabolite to prevent tumor formation and metastasis in the physiological tissue environment, e.g. in the prostate. In a

confirmed that the anti-migratory properties exerted by this steroid are mediated by ERβ. E2 was not able to mimic the anti-migratory effects of 3β-Adiol, again supporting the notion that the two ligands differentially activate their natural receptor.

The robust effects of 3β-Adiol on PC cell adhesion and against PC cell proliferation and migration suggested us to verify whether these activities were also accompanied by an anti-invasion ability. Thus, we tested the effect of 3β-Adiol on PC3-Luc cell invasion of a reconstituted basement membrane (Matrigel). For these experiments, on the basis of preliminary studies, we have chosen the 10 nM 3β-Adiol concentration. PC3-Luc cells spontaneously form cell aggregates in Matrigel, when prepared by the hanging drop technique (Fig. 4D), but the cells were not able to invade the Matrigel in normal conditions (Fig. 4D, see RPMI + vehicle). Instead, PC3-Luc cells exposed to 5% CS-FBS actively left the aggregate and invaded the Matrigel preparation (Fig. 4D, see RPMI + 5% CS-FBS + vehicle); 3β-Adiol (10 nM) strongly decreased cell migration through the Matrigel (Fig. 4D, see RPMI + 5% CS-FBS + 3β-Adiol).

**Figure 4** Effects of 3β-Adiol ICI 182,780 on cell migration and cell invasion. PC3-Luc cells were treated with either increasing concentration of 3β-Adiol (1–10 nM) (A) or 17β-estradiol (E2) (1–10 nM) (B) for 48 h; cells were then detached and transferred to Boyden’s Chamber. Bars represent the number of migrated cells counted in at least five independent wells for each group in the treated samples versus vehicle. The results of three separate experiments are presented as the mean ± s.d. Each experimental group was composed of 12 samples. Statistical analysis was performed by one-way ANOVA followed by Dunnett’s multiple comparison tests; **P<0.01 versus vehicle. (C) PC3-Luc cells were treated for 48 h with 3β-Adiol (10 nM) and ICI 182,780 (1 µM) either alone or in combination; cells were then detached and transferred to Boyden’s Chamber. Bars represent the number of migrated cells counted in at least five independent wells for each group in the treated samples versus vehicle. The results of three separate experiments are presented as the mean ± s.d. Each experimental group was composed of 12 samples. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison tests; **P<0.01 versus vehicle and §P<0.05 versus 3β-Adiol. (D) Matrigel invasion assay. Aggregates of PC3-Luc cells were exposed for 48 h to a RPMI + vehicle RPMI + 5% CS-FBS + vehicle, or RPMI + 5% CS-FBS + 3β-Adiol (10 nM). Images are representative examples of five independent experiments, and the same image is shown at two magnifications.
preliminary experiment, we set the conditions for an orthotopic implant of PC3-Luc cells in the mouse prostate and for the BLI analysis. To this purpose, 10^6 cells were injected into the prostate of BALB/c nu/nu nude mice, and tumor growth was followed weekly for 5 weeks. Tumor formation was consistent and initially localized in the correct anatomic area, while subsequently the luminescent cells spread out from the prostate to invade especially the peritoneal area (Supplementary Figure 5, see section on supplementary data given at the end of this article). Since we aimed at detecting the effects of 3β-Adiol on tumor progression and metastasis, we decided to use a smaller number of (10^3 cells) PC3-Luc cells orthotopically implanted into the prostate of 30 animals to test the effects of 3β-Adiol. Animals were divided into two treatment groups: one group was daily s.c. injected with 2.5 mg/kg 3β-Adiol and the other group with the corresponding amount of vehicle. Tumor growth was followed for 4 weeks by optical imaging, and after killing, we evaluated primary tumor weight and the invasion to adjacent seminal vesicles (i.e. early stage of metastasis formation; Fig. 6). Both tumor weight and invasion of seminal vesicle were significantly impaired by the hormonal treatment, again showing that 3β-Adiol is able to counteract PC progression and metastasis spreading from mouse prostate. As control, we measured serum levels of 3β-Adiol and DHT by mass spectrometry analysis; we found that treatment was able to raise the level of 3β-Adiol from 0.082 pg/μl (±0.01245 S.E.M.; vehicle group) up to 3.1 pg/μl (±0.3523 S.E.M.; 3β-Adiol group) that approximately corresponds to a blood concentration of 9 nM, while DHT levels were found to be 0.073 pg/μl (±0.01291 S.E.M.) and 0.16 pg/μl (±0.01869 S.E.M.) for vehicle- and 3β-Adiol-treated groups respectively.

Thus, our in vivo data were clearly demonstrating an anti-proliferative activity of 3β-Adiol that was shown to be relevant also on fully established neoplasia. In addition, 3β-Adiol seemed to counteract migration also in vivo as pointed out by the reduced number of seminal vesicles invaded by the PC3-Luc cells.

**Discussion**

In the present paper, we have analyzed the ability of the androgen derivative 3β-Adiol to decrease the aggressiveness of PC as measured by in vitro and in vivo progression, adhesion, and migration assays. To our knowledge, this is the first evidence showing in vivo that 3β-Adiol shows anti-progression and anti-metastatization activities. Anti-proliferative and anti-migratory activities of 3β-Adiol were previously reported in transformed cells in culture (Weihua et al. 2002a,b, Imamov et al. 2004, Guerini et al. 2005, Koehler et al. 2005). Here, we demonstrated that 3β-Adiol is able to reduce tumor progression of early-stage PC in a xenograft model, and to reduce the tumor growth and metastasis in established engrafted tumors.
This is likely due to the ability of 3β-Adiol to significantly modify several cell transformation parameters including adhesion, proliferation, migration, and invasion. Together, these in vivo and in vitro results strongly indicate that, in PC, 3β-Adiol counteracts the biological actions of its androgenic precursors testosterone and DHT, which are known to exert opposite and deleterious effects on the same tumor parameters, by favoring PC proliferation and invasiveness (Zhu & Imperato-McGinley 2009). This functional antagonism of 3β-Adiol appears to be molecularly independent from the activation of the androgenic pathway (Weihua et al. 2001, 2002a,b, 2003). In fact, AR is not expressed by PC3-Luc cells (Bonaccorsi et al. 2003), and 3β-Adiol is unable to bind to it. Moreover, all beneficial effects of 3β-Adiol are neutralized by the anti-estrogen ICI 182,780 and by the ERβ antagonist R.R'-THC. ERβ silencing using specific shRNAs against this receptor (Arnold et al. 2007, Treeck et al. 2010) also completely abolished the anti-proliferative/pro-adhesive effects of 3β-Adiol in PC3-Luc cells. This strongly suggests that the action of 3β-Adiol is mediated, at the molecular levels, by the estrogenic pathway. Since PC3-Luc cells express 3β-Adiol, but not ERα, it is likely that the β isoform is the molecular target of 3β-Adiol anti-proliferative and anti-invasive actions. Surprisingly, E2 was totally ineffective on the same parameters modified by 3β-Adiol, suggesting the existence of different ligand-selective pathways of ERβ in PC3 cells. Future studies are needed to unravel the molecular mechanism underlying this differential signaling. Considering that both E2 and 3β-Adiol are able to bind and activate the genomic function of ERβ in PC cancer cells (E2 binds to the receptor with 10–20 times higher affinity; Guerini et al. 2005), in order to explain a differential effect, one might hypothesize that a differential Helix 12 conformation imposed by the two ligands leads to a differential co-regulator complex recruitment at the regulatory regions of the target genes. A new concept is arising from our observations: the androgenic and estrogenic signals in the prostate may be generated by a fine equilibrium of the overall expression of several catabolic enzymes and nuclear steroid receptors as well as by the levels of the circulating and locally produced steroids. It is conceivable that a deregulation of this equilibrium (androgenic: AR bound either to testosterone or DHT; estrogenic: ERα and/or ERβ bound either to E2 or 3β-Adiol, either in homomeric or heteromeric dimers with the two alternative ligands) may be implicated in progression and invasiveness of PC. Therefore, at least in the early stages of PC, 3β-Adiol formation may shift the equilibrium versus a slowing down of progression and invasiveness of the tumor cells. This exciting hypothesis has strong support in clinical observations, demonstrating that genetic alteration of several enzymes involved in androgenic steroid metabolism is linked to hereditary and sporadic PC susceptibility (Chang et al. 2002, Steckelbroeck et al. 2004, Bauman et al. 2006, Cunningham et al. 2007, Neslund-Dudas et al. 2007, Park et al. 2007, Ross et al. 2008, Beuten et al. 2009, Mindnich & Penning 2009). It has been established that enzyme responsible for 3β-Adiol formation from the DHT in prostate is AKR1C1; in fact, AKR1C1:AKR1C2 (responsible for 3α-diol formation from the DHT) transcript ratio fells in PC; thus, the data here presented provide clear explanations of these observations, since the reduction of 3β-Adiol levels in favor of 3α-Adiol may be a risk factor for PC in these patients, because the 3β-Adiol ‘protective’ effect will obviously disappear. Moreover, differences in AR:ERβ transcript ratio were maintained in PC, suggesting that these classes of cancer may be more responsive to an ERβ agonist or SERM than to androgen ablation (Bauman et al. 2006).

It has also to be underlined that, on the basis of our results, the classical androgen depletion therapy widely utilized to treat PC should be re-evaluated. The complete removal of androgens, which can be obtained using GnRH analog treatments, would also remove 3β-Adiol, the agent that exert a protection against PC cell proliferation and metastasis formation. It must be noted that the classical side effects of these drugs are generally related to estrogen deficiency that occurs as a result of treatment. These are often considered the results of E2 deprivation, but the estrogenic effects of 3β-Adiol might contribute to these systemic alterations (Freedland et al. 2009). Therefore, the protection of 3β-Adiol will be preserved using AR antagonists only. A retrospective analysis should thus be conducted to determine whether the androgen-insensitive PC may become more aggressive because of the protection offered by 3β-Adiol is eliminated by the GnRH analog-based therapy.

We believe that several clinical and therapeutically relevant implications derive from the data obtained in the present study. For example, some studies reported beneficial effects of SERMs on PC development (Neubauer et al. 2003), and our data prove that 3β-Adiol has to be considered an ‘endogenous natural SERM’; this indicates that 3β-Adiol might be applied against PC growth and metastasis in untreatable androgen-independent PC. Thus, in future clinical perspective, 3β-Adiol could be evaluated as a candidate drug for human PC treatment, since it has no androgenic activity and cannot be a source of androgens.
Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0032.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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