Activation of membrane androgen receptors potentiates the antiproliferative effects of paclitaxel on human prostate cancer cells

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Abstract

Genomic signaling mechanisms require a relatively long time to get into action and represent the main way through which steroid hormones affect target cells. In addition, steroids may rapidly activate cellular functions by non-genomic signaling mechanisms involving membrane sites. Understanding in depth the molecular mechanisms of the non-genomic action represents an important frontier for developing new and more selective pharmacologic tools for endocrine therapies. In the present study, we report that membrane-impermeable testosterone-bovine serum albumin (BSA) acts synergistically with paclitaxel in modifying actin and tubulin cytoskeleton dynamics in LNCaP (androgen sensitive) and DU-145 (androgen insensitive) human prostate cancer cell lines. In addition, coinubcation of either cell line with testosterone-BSA and paclitaxel induced inhibition of cell proliferation and apoptosis. Finally, in vivo experiments in LNCaP and DU-145 tumor xenografts in nude mice showed that both agents decrease tumor mass, whereas testosterone-BSA enhances the effect of paclitaxel. Our findings suggest that chronic activation of membrane androgen receptors in vitro and in vivo facilitates and sustains for a longer time the antitumoral action of cytoskeletal acting agents. [Mol Cancer Ther 2006;5(5):1342–51]

Introduction

Steroid action is accomplished through two main pathways (reviewed in ref. 1): (a) After binding to intracellular receptors, the complex dimerizes, translocates to the nucleus, binds to specific regions of steroid response elements, and induces modulation of specific steroid-sensitive genes. This action, involving DNA transcription and RNA translation, takes time to be accomplished (>2 hours) and is sensitive to blockers of transcription and translation (2). (b) Steroids act through putative membrane sites, triggering the activation of specific cytoplasmic signaling cascades, including Ca2+ mobilization, and phosphorylation of a number of kinases (1). This action occurring within minutes and insensitive to blockers of transcription and translation is also present in cells lacking intracellular receptors, and in most cases, it is insensitive to specific steroid antagonists.

Rapid effects of androgens have been identified on a number of cells bearing or not intracellular receptors, including osteoblasts (3), macrophages (4), T lymphocytes (5), prostate cancer (6), Sertoli (7), breast (8, 9), and vascular endothelial cells (10). By the use of nonpermeable fluorescent testosterone analogues [FITC-bovine serum albumin (BSA)–bound testosterone], we have identified membrane binding on prostate cancer cells and characterized it pharmacologically as a putative membrane testosterone receptor (6). Membrane androgen receptors (AR) are preferentially expressed in prostate cancer compared with prostate hyperplasia or nonhyperplastic tissue (11) and are directly correlated to the Gleason’s sum of the tumor (12).

Recently, we have reported that long-term exposure of prostate cancer cells to testosterone-BSA results in decreased adhesion, invasion, and migration of cancer cells as well as in induction of apoptosis (13). In addition, treatment of human cell-derived tumor-bearing athymic mice with testosterone-BSA resulted in a 60% reduction of tumor mass. Activation of membrane androgen binding sites induced rapid Ca2+ movements (7, 14–16), triggering of specific signaling cascades (17, 18) and finally actin cytoskeleton rearrangement modifies cell-substratum adhesion, controlling a number of cell functions, such as motility, division, and secretion (19–21). These findings support the hypothesis of possible interactions of membrane AR activators (testosterone-BSA) with other cytoskeletal-acting drugs, such as paclitaxel. Microtubule acting drugs (paclitaxel and docetaxel) were effectively used for the management of a number of cancers, where other chemotherapeutics were not very efficient. Recently, two studies have shown that in advanced, refractory prostate cancer, low doses of these agents, given weekly or every 3 weeks, improve survival,
reduce pain, and decrease the levels of prostate-specific antigen (22, 23), although with significant side effects. In view of the effectiveness of cytoskeletal acting agents in the treatment of prostate cancer, it is of interest to consider therapeutic regimes of these agents in association with possible potentiators of their efficacy, in view of a possible reduction of its dosing, resulting in diminished side effects.

In the present study, we have tested the hypothesis that activation of membrane ARs may increase the potency on the antiproliferative effect of paclitaxel, taken as an example of cytoskeletal modifiers, in prostate cancer cells. Our results, both in vitro and in vivo, show that the combination of membrane-impermeable testosterone-BSA and paclitaxel can further reduce the growth of prostate tumors in an additive way. Furthermore, our data suggest that addition of testosterone-BSA enhances the effects of cytoskeletal acting agents, which may then act with the same efficacy at 30 times lower concentrations.

Materials and Methods

Cell Culture
LNCaP and DU-145 cells (DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 (Life Technologies-Invitrogen, Paisley, United Kingdom), 10% fetal bovine serum 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) and paclitaxel were purchased from Sigma (St. Louis, MO) and used dissolved in PBS buffer. Before each experiment, a new solution of BSA conjugate was prepared and subjected to DCC treatment (0.05 mg/mL dextran and 50 mg/mL charcoal) for 30 minutes to remove any potential contamination with free testosterone (13). We assayed routinely culture medium for the presence of free testosterone with a specific RIA method with negative control group, 100 μg/mL charcoal) for 30 minutes to remove any potential contamination with free testosterone (13). We assayed routinely culture medium for the presence of free testosterone with a specific RIA method with negative results. Cell growth was assayed by the tetrazolium salt assay (24).

Assessment of the Polymerization State of Cytoskeletal Proteins
Monomeric (triton soluble) and polymerized (triton insoluble) actin and tubulin were assayed as previously described (25). Actin and tubulin were visualized with monoclonal anti-actin (Amersham-Pharmacia, Buckinghamshire, United Kingdom) or anti-β-tubulin (Sigma) antibodies and second horseradish peroxidase–coupled antibody (Chemicon International, Temecula, CA).

Visualization of Actin and Tubulin Cytoskeleton
Cells were grown on poly-l-lysine-coated coverslips. After addition of the different agents, actin- and tubulin-network were visualized by direct and indirect fluorescence microscopy, respectively. Cells were fixed with 4% formaldehyde in PBS for 5 minutes at room temperature, permeabilized with 0.5% Triton X-100, and incubated in blocking buffer (PBS, 0.5% Triton X-100, and 1% fish skin gelatin). Actin was stained with rhodamine-phalloidin (1:100), whereas microtubules were identified with an anti-β-tubulin antibody (1:150; clone TUB21; Sigma) and FITC-conjugated anti-mouse immunoglobulins (1:200). Specimens were analyzed in a Leica SP confocal microscope.

DNA Staining and Detection of Cell Cycle and Apoptosis
Cells were fixed with 3 mL of absolute ethanol (4°C for 1 hour), washed, and provided with 1 mL of a 50 μg/mL of propidium iodide in sodium citrate and 50 μL of a 10 μg/mL RNaseA solution. After 3 hours of incubation at 4°C, they were assayed by flow cytometry, using a Becton Dickinson FACSAarray apparatus (Becton Dickinson, Franklin Lakes, NJ) and analyzed with the CELLQuest (Becton Dickinson) and ModFit LT (Verify Software, Topsham, MN) software.

Semi-quantitative Measurement of Apoptotic Proteins
Measurement of proapoptotic and antiapoptotic proteins was made as described previously (26). The following antibodies were used: anti-human Bcl-2 monoclonal antibody (clone 124; DAKO, Glostrup, Denmark; 1:200); rabbit polyclonal anti-serum against Bax, Bak, Bcl-2, Bcl-xl, and Bad (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100); the anti-Fas (1:2,500) and anti-FasL (1:1,000; Transduction Laboratories, Lexington, KY). Secondary antibodies were goat peroxidase–conjugated anti-mouse IgG (Chemicon International; 1:10,000) or anti-rabbit IgG (Immunotech, Marseille, France; 1:4,000). For purposes of normalization, the blots were also stained with a monoclonal anti-actin antibody (1:400; Amersham-Pharmacia).

In vivo Experiments with Nude Mice
Male BALB/c-c nude mice (10 weeks old) were from Harlan (A. Pietro al Natisone, Italy). Animals were injected s.c. in the back with 5 × 10⁶ LNCaP or DU-145 cells diluted in Matrigel (Sigma) in a total volume of 0.1 mL. After 2 or 4 weeks for DU-145 and LNCaP cells, respectively, macroscopic tumors were developed. Then, testosterone-BSA, paclitaxel, or their combination, supplemented with the anti-androgen cyproterone acetate, diluted in PBS, was injected i.p. thrice per week, in a total volume of 0.5 mL. Tumor size was measured with a Vernier caliper weekly, and its weight was calculated by the formula 0.5a × b², where a is the long diameter, and b is the short diameter of the tumor (both in cm; ref. 27). The animals were sacrificed at the indication time. Tumors were excised, measured, fixed in formalin, and analyzed by a pathologist. Liver, prostate, and testes were analyzed by the same pathologist blindly for changes indicative of testosterone and/or paclitaxel action. The inhibitory rate of tumor growth was calculated according to the following formula (28):

\[ \text{inhibitory rate} = \frac{C(W_{1} - W_{0}) - T(W_{1} - W_{0})}{C(W_{1} - W_{0})} \]

where C is control group, T is the treated group, W₁ is the tumor weight before treatment, and W₀ is the weight after treatment.

Immunohistochemistry
Serial sections of tumors (3-μm thick) were cut from each paraffin block and layered on negatively charged (SuperFrost Plus) slides (Kindler O GmbH, Freiburg, Germany). Anti-β-tubulin antibody (1:150; clone TUB21; Sigma) and FITC-conjugated anti-mouse immunoglobulins (1:200) were used. Specimens were analyzed in a Leica SP confocal microscope.
Germany). One slide was stained with H&E 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; dilution 1:50). Fast red was used as chromogen, and Mayer’s hematoxylin was used for counterstaining. The indirect terminal deoxynucleotidyl transferase–mediated nick-end labeling enzymatic labeling technique was used to detect apoptotic activity of the tumors (in situ cell death detection kit with alkaline phosphatase; Boehringer, Mannheim, Germany). All tumors were analyzed blindly by the same pathologist.

Statistical Analysis
Statistical analysis was done by the use of appropriate test, using the SPSS (SPSS, Chicago, IL) computer program. Statistical significance was set to $P < 0.05$.

![Figure 1](image-url)

**Figure 1.** A, time course of actin and tubulin polymerization state in the presence of testosterone-BSA in DU-145 (left) and LNCaP (right) prostate cancer cells. Cells were incubated for different time periods with $10^{-7}$ mol/L testosterone-BSA. After 24 h of incubation, the medium was replaced with fresh, free of any agent. Triton soluble/triton insoluble ratio of actin and tubulin divided by the corresponding control values. Points, mean of three different experiments done in triplicate; bars, SE. B, organization of actin and tubulin cytoskeleton, in DU-145 (left) and LNCaP (right) cells treated with testosterone-BSA. Cells were incubated for 24 h with testosterone-BSA ($10^{-7}$ mol/L). Thereafter, they were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated with blocking buffer. Actin cytoskeleton was stained with rhodamine-phalloidin and microtubules using monoclonal antibodies recognizing $\beta$-tubulin. Specimens were analyzed in a Leica SP confocal microscope. Compare cytoskeleton rearrangements in control (arrowheads) and treated cells (arrows).

Results

**In vitro Effects**

Long-term Activation of Membrane Testosterone Receptors Modifies Cytoskeleton in LNCaP and DU-145 Prostate Cancer Cells. Previous results showed that long-term incubation of iAR-positive LNCaP and iAR-negative DU-145 cells with $10^{-7}$ mol/L testosterone-BSA or testosterone resulted in a dose-dependent and time-dependent inhibition of cell growth and induction of apoptosis (13). Additionally, short-term (10 minutes) incubation of LNCaP cells with testosterone-BSA or testosterone produced a potent but transient actin polymerization and microfilament reorganization, through activation of a specific signaling cascade (17). To assess the effect of long-term exposure of LNCaP and DU-145 cells to testosterone-BSA on the dynamics of cytoskeletal proteins, we have measured the ratio of nonpolymerized (triton soluble) to polymerized triton-insoluble actin and tubulin, after testosterone-BSA treatment, at a concentration $10^{-7}$ mol/L, inducing a submaximal...
effect on cell growth inhibition and apoptosis (13). As depicted in Fig. 1A, incubation of LNCaP cells for 24 hours with testosterone-BSA resulted in a significant decrease of actin polymerization (triton soluble/triton insoluble = 2.5 ± 0.08 over control; \( P < 0.001 \)), indicating a differential action of testosterone-BSA on actin dynamics for short-term and long-term treatment. In contrast, in DU-145 cells, no significant changes of actin polymerization were observed. When the incubation of cells was continued for another 48 hours, after changing the medium and withdrawing testosterone-BSA, the polymerization ratio of actin returned to control values in LNCaP cells. A similar, although less pronounced, effect was also found when tubulin polymerization was measured (Fig. 1A). Indeed, in LNCaP cells, a statistically significant decrease in tubulin polymerization by 1.3 ± 0.03 over control (\( P < 0.01 \)) was observed, after incubation with \( 10^{-7} \) mol/L testosterone-BSA. Thereafter, they were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated with blocking buffer. Actin cytoskeleton was stained with rhodamine-phalloidin and microtubules using monoclonal antibodies recognizing α-tubulin. Specimens were analyzed in a Leica SP confocal microscope. Compare cytoskeleton arrangement in control (arrowheads) and treated cells (arrows).

Testosterone-BSA Potentiates the Antiproliferative and Proapoptotic Effects of Paclitaxel on LNCaP and DU-145 Cells. The above findings suggest that testosterone-BSA, in addition to the reported short-term effects (6, 17), may induce long-term changes in cytoskeletal protein dynamics. We have, therefore, investigated whether paclitaxel, an agent known to significantly alter the stability of microtubules, could be potentiated by testosterone-BSA.

In LNCaP cells, paclitaxel increased the tubulin polymerization rate. When testosterone-BSA and paclitaxel were both applied to the cells, the effect of paclitaxel was enhanced (Fig. 2A). Cytoskeletal changes were also observed by confocal laser scanning microscopy (Fig. 1B). When DU-145 cells were treated with testosterone-BSA, a partial reorganization of actin and tubulin cytoskeleton was evident (pointed out by arrows in Fig. 1B). Microtubules are shown less structured and packed mainly at the cell periphery. Similar effects were noted in LNCaP cells after testosterone-BSA. When DU-145 and LNCaP cells were incubated with paclitaxel (Fig. 2B), extensive bundling of microtubules was observed. Actin cytoskeleton was also changed, and actin was redistributed and concentrated at distinct sites peripherally. Similar changes, but to a lesser extend, were observed when cells were coincubated with testosterone-BSA and paclitaxel.
microtubules, and testosterone-BSA could act in concert to affect the cytoskeleton dynamics and cancer cell growth. Incubation of LNCaP or DU-145 cells with varying concentrations of paclitaxel induced a dose-dependent inhibition of cell growth, with an IC₅₀ of 7.3 ± 0.5 nmol/L (data not shown). This effect persisted after replacing with fresh medium, in the absence of the agent and incubation for another 48 hours. In that case, paclitaxel IC₅₀ was lower (53.5 ± 2.6 nmol/L). Based on these findings, we used a fixed concentration of paclitaxel (10⁻⁸ mol/L) in all subsequent experiments. Furthermore, we have fixed the concentration of testosterone-BSA to 10⁻⁷ mol/L, based on our previous results (13), showing that testosterone-BSA acts as an antiproliferative agent in LNCaP and DU-145 cells with an IC₅₀ of 5 and 10 nmol/L, respectively, when it is continuously present, and of 46 and 100 nmol/L, respectively, when it is depleted after 24 hours.

Figure 3 (top) presents the effect of testosterone-BSA on LNCaP and DU-145 cell growth. As previously reported (13), testosterone-BSA (10⁻⁷ mol/L) induced a decrease of LNCaP cell growth by 49%, of control (P < 0.001). Paclitaxel (10⁻⁸ mol/L) on the other hand, decreased cell growth by 72% of control, and the combination of both agents further decreased the growth of LNCaP cells by 81%, indicating a significant additive effect of the agents on cell proliferation. Similar, although less pronounced, effects were observed in DU-145 cells (decrease of cell growth by 32%, 36%, and 51% in the presence of testosterone-BSA, paclitaxel, and their association, respectively; P < 0.001). An analysis of a dose-response of testosterone-BSA, paclitaxel, and their association at a constant ratio 1:9 (testosterone/paclitaxel) in DU-145 cells (Fig. 3, bottom) revealed a combinatorial index of 0.37, suggestive of a strong synergism between the two drugs (29). A similar result was also obtained in LNCaP cells (combinatorial index = 0.41; data not shown).

Incubation of cells with the combination of testosterone-BSA (10⁻⁷ mol/L) and paclitaxel (10⁻⁸ mol/L) increased apoptosis of LNCaP cells (Fig. 4), as reflected by the induction of apoptotic hypodiploid DNA (Fig. 4A). This effect was evident after 24 hours and significantly more pronounced after 24 + 48 hours, where ~45% of cells showed an apoptotic pattern. Detection of apoptosis-related proteins (Fig. 4B) indicates that testosterone-BSA induced the expression of Fas, at 24 and 24 + 48 hours, whereas a transient increase was also found after paclitaxel incubation at 24 hours. On the other hand, a significant increase of the proapoptotic Bad protein was observed after 24 hours of incubation with paclitaxel and testosterone-BSA. The levels of this protein has returned to normal values after withdrawal of the agents (24 + 48 hours of incubation). Similar results were obtained in DU-145 cells (data not shown).

In vivo Effects

Testosterone-BSA and Paclitaxel Act in Concert to Decrease Tumor Mass of LNCaP- and DU-145-Induced Tumors in Nude Mice. Our previous findings have
shown that LNCaP-inoculated nude mice respond positively to treatment with testosterone-BSA (8 mg/kg body weight) by decreasing tumor mass and inducing apoptosis of tumor cells (13). Additionally, the in vitro effects presented above suggest that testosterone-BSA–induced rearrangements of the actin cytoskeleton and paclitaxel (acting on the tubulin cytoskeleton) may act in combination, inhibiting cell growth. We have, therefore, tested the effects of the simultaneous administration of testosterone-BSA (8 mg/kg body weight) and paclitaxel in vivo, using the same experimental model as previously described (i.e., LNCaP- or DU-145-inoculated nude mice treated every other day i.p. with either drug or their combination).

In all cases, including control nontreated animals, we have added to animal treatment cyproterone acetate, a pure anti-androgen, at a dose of 52 mg/kg body weight, to counteract both the effects of endogenous androgen and the effects of a possible dissociation of testosterone from the BSA molecule.

In a preliminary experimental series, LNCaP-and DU-145-inoculated mice were treated (thrice per week for 1 month) with testosterone-BSA (8 mg/kg), paclitaxel (8 mg/kg), or their combination. A decrease in tumor mass was observed in the animals treated either with testosterone-BSA or paclitaxel alone, and their effect was enhanced by their combination (data not shown). Consequently, mice bearing LNCaP and DU-145 tumors were treated (thrice per week for 1 month) with three different concentrations of paclitaxel (4, 0.8, and 0.08 mg/kg body weight), and a dose-dependent decrease of tumor mass was induced (Figs. 5A and 6A). Paclitaxel was less effective in DU-145-induced tumors, as these cells were more aggressive and induced larger tumors in mice. The addition of testosterone-BSA (8 mg/kg) induced a stronger tumor reduction compared with paclitaxel alone. The addition of testosterone-BSA resulted in 30 times greater potency of paclitaxel on LNCaP tumor growth inhibition (IC₅₀ = 0.8 compared with IC₅₀ = 27.1 mg/kg body weight; Fig. 5A), whereas in DU-145 tumors (Fig. 6A), the effect of paclitaxel was enhanced to a lesser extend (IC₅₀ = 0.15 and 0.05 mg/kg body weight, respectively, in the absence or in the presence of testosterone-BSA). Finally, in view of these results, we further treated LNCaP- or DU-145-inoculated animals with a fixed concentration of paclitaxel (0.8 mg/kg body weight), and/or testosterone-BSA (8 mg/kg body weight) every other day, for prolonged time periods, to test the efficacy of each agent alone or in combination.

Figures 5B and 6B depict the inhibitory rate of tumor growth for each agent. This index provides a normalized ratio of tumor weight change at a given time point compared with the weight change of control (nontreated) animals. LNCaP tumor-bearing animals responded to either testosterone-BSA or paclitaxel administration. Testosterone-BSA had an inhibitory rate efficacy of about 40%, which...
remained stable throughout the observation period (70 days). Paclitaxel had an efficacy of 50% attained at about 25 days and remaining constant thereafter. In addition, coadministration of paclitaxel and testosterone-BSA showed a strong additive effect, reaching an inhibitory efficacy of 92% at 1 month, which remained constant thereafter. In contrast, in DU tumor-bearing animals (Fig. 6B), the inhibitory efficacy of either paclitaxel or testosterone-BSA alone reached a plateau (~50% in either case) from days 10 to 40 and declined thereafter, attaining only 15% at day 65. This can be attributed to the aggressiveness of the DU-145 cell–induced tumors. Interestingly, coadministration of both drugs resulted in an additive (inhibitory efficacy ~70%) and sustained effect during the whole observation period. In this respect, we have concluded that coadministration of testosterone-BSA with paclitaxel potentiates the antitumoral action of the later drug, preserving its action and efficacy for a longer time period.

The histologic analysis of untreated tumors showed mitoses predominating at the periphery of the tumors and scarce apoptotic bodies (Figs. 5 and 6, D and E), which were slightly decreased in testosterone-BSA–treated animal tumors. Paclitaxel decreased the number of mitoses, especially in tumors with simultaneous testosterone-BSA treatment. In addition, testosterone-BSA increased the number of apoptotic cells, an effect that was more pronounced in LNCaP tumors. Apoptosis was found also slightly increased in the paclitaxel treated animal tumors, whereas treatment with paclitaxel together with testosterone-BSA evoked the greatest apoptosis both in LNCaP and DU-145 tumors.

In conclusion, treatment of animals with both paclitaxel and testosterone-BSA decreased mitoses and induced apoptosis, explaining the higher tumor mass regression in the presence of their combination and indicating an additive action of these agents in vivo. Detection of membrane ARs in animals treated for 45 days with testosterone-BSA confirmed their presence (data not shown), suggesting that no down-regulation of the receptors occurs after a prolonged treatment. Analysis

![Figure 5](image-url)

**Figure 5.** In vivo effects of testosterone-BSA and paclitaxel in LNCaP tumor-bearing nude mice. **A,** dose-effect of paclitaxel administration (every other day, i.p.) alone or with 8 mg/kg body weight of testosterone-BSA. Tumor mass after 4 wks of treatment compared with the corresponding control, taken as 100% in all cases. Points, mean of tumor mass compared with control animals; bars, SE. Each group consisted of five mice. In all groups, a dose of 52 mg/kg cyproterone acetate was coadministered to prevent any interaction with intracellular AR. **B,** inhibitory rate of animals (five per group) treated with 8 mg/kg body weight testosterone-BSA (8 mg/kg body weight), paclitaxel (0.8 mg/kg body weight), or their combination, supplemented with 52 mg/kg cyproterone acetate. The inhibitory rate of tumor growth was calculated according to Zhou et al. (28). Points, mean of each group; bars, SE. **C,** representative histologic preparations from each animal group. **D** and **E,** MIB-1 staining of tumors and terminal deoxynucleotidyl transferase–mediated nick-end labeling assay, respectively. In terminal deoxynucleotidyl transferase–mediated nick-end labeling staining, apoptotic cells are stained deep purple. In MIB-1 staining, dividing cells are intensively stained.
of liver, prostate, and testes did not show any evidence of testosterone stimulation (data not shown), indicating the absence of testosterone dissociation from its complex with BSA.

Discussion

The classic way by which steroid hormones program the target cells for complex functions involves genomic signaling mechanisms and provide cells with the tools to accomplish their tasks. These mechanisms require a relatively long time to get into action and could determine the medium-term and long-term fate of the cell (2). In contrast, non-genomic signaling may represent a mechanism through which steroid receptors rapidly activate the cellular functions needed for adaptation to dynamic changes in the surrounding milieu. These mechanisms would rapidly enable the cell to use tools that are already present and become functionally activated or repressed at an extremely short time period. Therefore, understanding in depth the molecular mechanisms through which these actions are exerted represents an important frontier to engineer new and more selective pharmacologic tools for endocrine therapies (1). Nevertheless, until recently, no data existed about long-term effects via activation of membrane steroid receptors.

Prostate cancer seems a tissue of choice for the detection of non-genomic actions of androgens, especially because androgen independence represents a major therapeutic problem. In this tissue, a preferential expression of membrane ARs has been detected (11) compared with noncancer cells, correlating to the Gleason's grade (12), and therefore directly proportional to the aggressiveness of the tumor.

In a previous study (13), we have reported that activation of membrane testosterone receptors decreases cell proliferation and motility, invasiveness, and adhesion and induces apoptosis in vitro. It seems that the decrease of cell division/viability and the induction of apoptosis is related to actin cytoskeleton rearrangements and a specific
signaling cascade (6, 17). In addition, we have reported that long-term (30 days) activation of testosterone membrane sites in nude mice bearing LNCaP tumors induces tumor regression through apoptotic mechanisms (13). In the current work, we present evidences that activation of testosterone membrane sites in nude mice bearing DU-145 androgen-insensitive tumors has similar antitumoral effects. These actions could not be attributed to ARs, which are absent from this cell line, but to a specific activation of membrane androgen sites. These sites seem different in nature from classic receptors, as they are only partially recognized by an antibody (N-29) directed against the NH$_2$-terminal part of the AR but not by the C-19 antibody against the hormone-binding COOH terminus (9). In addition, membrane ARs seem to be G-protein coupled, a result not in line with the classic AR mode of action (see ref. 30 for a review). We have further addressed the question whether the combination of cytoskeleton modifiers, such as paclitaxel and activators of membrane ARs (testosterone-BSA), may act in concert in inducing prostate cancer cell regression. Indeed, cytoskeleton modifiers (docetaxel) have been recently shown to increase survival, reduce pain, and decrease the levels of prostate-specific antigen of refractive prostate cancer (22, 23). Therefore, in view of the efficacy of cytoskeleton modifiers in the treatment of the disease, it might be interesting to consider therapeutic regimes with this agent, combined with other compounds potentiating its efficacy, while reducing its dosing and side effects. Intermittent modes of administration of the drug have been recently introduced in clinical practice, permitting less complications (31). However, the benefit of this dosing was rather limited in prostate cancer patients (22, 23). To address this question, we did in vitro tests, in which paclitaxel, testosterone-BSA, and their combination were applied to cells for 24 hours and withdrawn thereafter and measured their effects on the cytoskeleton and on cell proliferation and apoptosis. In addition, in in vivo experiments, we have grafted nude mice with DU cells and, after tumor formation, we have treated them with either agent or their combination. Both in in vitro and in vivo experiments, although we had no indication of liberation of free testosterone, we have supplemented cell cultures or animals with the anti-androgen cyproterone acetate, which per se had no effect on cell growth or apoptosis. Our data provide experimental evidences that activators of membrane ARs could be a good candidate for a more specific therapy, supplementing current therapeutic regimes. We have shown that the action of paclitaxel can be enhanced with coadministration of testosterone-BSA, a compound deprived of side effects. Nevertheless, until the identification of membrane ARs and the synthesis of specific (nonpeptide?) agonists, we suggest the simultaneous use of anti-androgens to counteract the adverse effects of a possible dissociation of testosterone from the complex.

Membrane AR Activators Potentiate Paclitaxel Effects

We have previously reported that in other systems (32) and in prostate cancer cells (13), the decrease of cell proliferation combined with modifications of cytoskeleton dynamics could lead to apoptosis. Indeed, apoptosis is also triggered in prostate cancer cells both in vitro and in vivo, after testosterone-BSA treatment. A major implication of the Fas/Fasl system was found, indicating that the Fas system might be implicated in the proapoptotic effect of the conjugate. In addition, the antiapoptotic proteins Bcl-2 and Bcl-xL were found increased as an initial rescue response of the cells to the proapoptotic challenge, whereas apoptotic Bad levels were found increased after paclitaxel (33). It is worth noticing that testosterone-BSA and paclitaxel affect cell growth by a different mechanism, the former by directly inducing apoptosis, whereas the latter by inducing mitotic arrest and thereafter cell death.

Prostate cancer is the commonest diagnosed disease in Western male populations and the second leading cause of cancer-related deaths (34). The disease usually starts as an androgen-dependent tumor and evolves to androgen insensitivity. For the time-been, no specific therapy of prostate cancer has been proposed: surgical ablation of the tumor is usually done followed by anti-androgen and/or chemotherapy, with poor results. To have a realistic chance to tackle the poor outcome of the advanced disease, the identification of alternative therapeutic approaches is necessary. Recently, docitaxel chemotherapy has been proven a good candidate, in spite of increased adverse reactions of the drug (22, 23). Our data provide experimental evidences that activators of membrane ARs could be a good candidate for a more specific therapy, supplementing current therapeutic regimes. We have shown that the action of paclitaxel can be enhanced with coadministration of testosterone-BSA, a compound deprived of side effects. Nevertheless, until the identification of membrane ARs and the synthesis of specific (nonpeptide?) agonists, we suggest the simultaneous use of anti-androgens to counteract the adverse effects of a possible dissociation of testosterone from the complex.

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