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Inhibitory Effects of Nordihydroguaiaretic acid (NDGA) on the IGF-1 Receptor and Androgen Dependent Growth of LAPC-4 Prostate Cancer Cells

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Abstract

Background—Nordihydroguaiaretic acid (NDGA) is an inhibitor of the IGF-1 receptor (IGF-1R) in breast and other cancers, and concomitantly inhibits tumor growth both in cultured cells and animals. The current study evaluates the effect of NDGA on the androgen-stimulated growth of human prostate cancer cells.

Methods—LAPC-4 prostate cancer cells in tissue culture were androgen starved for 3 days, 1 nM dihydrotestosterone (DHT) and other androgens were then added for up to 7 days, and cell proliferation measured. IGF-1R protein expression was measured by western blot, and IGF-1R mRNA expression by quantitative PCR. IGF-1R receptor kinase activation was measured by ELISA.

Results—After 7 days, LAPC-4 growth was doubled by 1 nM DHT. NDGA had a rapid effect to inhibit IGF-1R autophosphorylation induced by IGF-1. DHT increased the expression of IGF-1R protein and mRNA levels. Maximal IGF-1R protein levels were observed 3 days after the addition of androgen. In addition, NDGA, at 10 μ M or less, inhibited DHT-induced proliferation in both cells grown in plates and cells grown in soft agar. Androgen receptor (AR) studies by FRET revealed that NDGA had no conformational effects on the AR in response to ligand.

Conclusions—NDGA blocks the DHT-induced growth of LAPC-4 prostate cancer cells by several mechanisms including rapid inhibition of the IGF-1R kinase, and a dose-dependent inhibition of androgen stimulation of IGF-1R expression. Clinical studies of this agent will determine its efficacy in the setting of androgen-dependent prostate cancer.

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Keywords

insulin-like growth factor 1 receptor; insulin-like growth factor 1; prostate cancer; tyrosine kinase; gene expression; NDGA

INTRODUCTION

A hallmark of human prostate cancer is the tumor's sensitivity to androgen stimulation of growth, a process that relies on a variety of ligand directed and co-stimulatory mechanisms (1;2). A growing body of literature supports the hypothesis that non-androgen signaling mechanisms within prostate cancer cells can also regulate tumor cell proliferation, many of which are implicated in the emergence of progressive disease in a testosterone-depleted milieu. Certain of these non-androgen mechanisms appear to include cell surface tyrosine kinases including the insulin like growth factor-1 receptor (IGF-1R) (3–6).

The IGF-1R and its ligands, IGF-1 and IGF-2, play a key role in regulating growth, resistance to apoptosis, and invasion in a variety of human cancers (7–10). A number of studies have established a role for the IGF system in prostate cancer. First, clinical and epidemiological data indicate that elevated serum IGF-1 levels are a risk factor for prostate cancer (11;12). Second, the IGFs increase the growth of prostate cancers in cultured cells (13;14). Third, abrogation of the IGF-1R via anti-sense suppresses growth and invasion by rat prostate cells *in vivo* (4). Further, the progression of some androgen sensitive cell lines to androgen independent growth in xenografts, is accompanied by an increased expression of both IGF-1 and IGF-1R (5).

Meso-nordihydroguaiaretic acid (NDGA), a butanediol, is a compound isolated from *Larrea tridentata*, more commonly known as chaparral or the creosote bush. *L. tridentata* grows in the southwestern United States and Mexico, and extracts of the leaf and/or stem have been taken orally by the Pima Indians and other cultures in these regions to treat various conditions (15). Prior studies from our laboratory have demonstrated that purified NDGA inhibits the IGF-1R tyrosine kinase (16–18). In breast cancer and neuroblastoma cells, NDGA both inhibits growth in tissue culture and reduces tumorigenesis (17;19) in animals. We have recently reported that this receptor is expressed in nearly all prostate cancers and metastases (20). Accordingly the IGF-1R is a potential target in these cancers.

In the present study, the effects of NDGA on the growth and proliferation of prostate cancer cells stimulated with androgen are evaluated. The human prostate cancer cell line, LAPC-4, was utilized because of the absence mutations in either the androgen receptor (AR) or PTEN, and its sensitivity to androgen stimulation of proliferation (21;22). We now report that, in LAPC-4 cells grown in tissue culture, NDGA attenuates growth in concert with both direct inhibition of the IGF-1R tyrosine kinase and inhibition of androgen-stimulated expression of the IGF-1R protein.

METHODS AND MATERIALS

NDGA and IGF-1 were from Insmed Corp (Richmond, VA). The following were purchased: antibodies against the IGF-1R (C-20), phosphospecific antibodies recognizing phosphotyrosine (PY20), and HRP-conjugated anti-phosphotyrosine antibody (PY20HRP) were from Santa Cruz Biotechnology (Santa Cruz, CA); alpha IR3, a monoclonal antibody against the IGF-1R, was from CalBiochem (San Diego, CA); phosphospecific antibody pIGF-IR (Y1131) was from Cell Signaling (Beverly, MA); methyltrienolone (R1881) was from Perkin Elmer Life Sciences, Inc (Boston, MA) and coated protein A Sepharose CL4B (Amersham Biosciences, Sweden). Anti-insulin receptor antibody (CT-3) was from Neo Markers (Fremont, CA). Unless specified, all other reagents were from Sigma (St. Louis, MO).

Growth studies of LAPC-4 prostate cancer cells in plates

LAPC-4 prostate cancer cells were maintained at 37°C, 5% CO₂ in phenol-free RPMI +10% FCS RPMI. Steroid-free medium consisted of phenol-free RPMI supplemented with 10% dextran-coated, charcoal-treated serum (10% CDSS RPMI). LAPC-4 cells were incubated in this steroid-free 10%CDSS RPMI for 3 days prior to plating in 96-well plates (5 × 10³ cells/well). Cells were allowed to adhere overnight and were then treated with androgens and various concentrations of NDGA with DMSO as a vehicle control. The medium with androgens and inhibitors was refreshed on day 3. The plates were harvested on day 7 by inverting the microplate onto paper towels with gentle blotting to remove growth medium without disrupting adherent cells and freezing them at -80°C for at least 30 minutes. LAPC-4 prostate cancer cell growth was determined using either the CyQuant cell proliferation assay (Molecular Probes, Eugene, OR) or by the BCA assay (Pierce, Rockford, IL). Cell proliferation was calculated as the percent of content versus control cells at day 0.

Growth studies of LAPC-4 prostate cancer cells in soft agar cells

LAPC-4 cells were androgen starved, as described above, for 3 days. Cells were plated in 96-well plates in 0.2% agar layer over 0.4% base agar layer as follows: To prepare 0.4% base agar layer, 0.8% agar solution at 37°C was mixed with 2X 10%CDSS RPMI. Cells were harvested and resuspended in culture medium (10%CDSS RPMI). For each well to be plated, 20 µl of 0.8% agar was mixed with 40 µl cells and 20 µl 2X 10%CDSS RPMI. When the top layer solidified, 1 nM dihydrotestosterone (DHT) was added in 50 µl of 1X 5%CDSS RPMI. NDGA was applied in 50 µl of 1X media the following day. Cells were refreshed on day 3. Cells were grown for 6 days at 37°C, 5% CO₂. Experiments were terminated on day 6, by aspirating the liquid culture and solubilized in 3 M guanidine isothiocyanate at 45°C for 1 hour. The CyQuant cell proliferation assay was then employed.

Ligand-stimulated IGF-IR autophosphorylation in LAPC-4 prostate cancer cells

For IGF-1R autophosphorylation, cells were transferred into 10%CDSS RPMI 3 days prior to the experiment. The cells were then plated in 6-well plates in presence of 1 nM DHT. At the end of either a 1- or 3-day incubation period, cells were serum-starved for 2 hours. NDGA was dissolved in DMSO and diluted with culture medium before being added to cells for 1.5 hour at 37°C. The final concentration of DMSO during the incubation was 0.3%.

Cells were then stimulated with 3 nM IGF-I for 10 minutes at 37°C. Reactions were terminated by rapidly aspirating medium and washing cells 3 times with ice cold phosphate-buffered saline (PBS) at 4°. Cells were harvested and solubilized in 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 2 mM vanadate for 1 hour at 4°C. Protein was determined by BCA assay. IGF-IR autophosphorylation was determined by ELISA (17). Briefly, 10 µg lysate protein was added to triplicate wells in a 96-well plate coated with monoclonal antibody to the IGF-IR (αIR3; 2 µg/ml), and incubated for 18 hours at 4°C. Plates were washed 5 times, and then HRP-conjugated anti-phosphotyrosine antibody (0.3 µg/ml), diluted in 50 mM HEPES, pH 7.6, 150 mM NaCl, 0.05% Tween-20, 1 mM PMSF, 2 mM vanadate and 1 mg/ml bacitracin, was added for 2 hours at 22°C. Plates were washed 5 times prior to color development with TMB substrate, which was terminated with 1.0 M H₃PO₄. Values for receptor autophosphorylation were determined by measuring absorbance at 450 nm.

IR/IGF-IR expression studies

Cells were androgen-starved in 10% CDSS RPMI, as described above, prior to their plating in 100 mm dishes with various doses of androgens and/or NDGA. At the end of the incubation period, cells were washed twice with PBS and solubilized with lysis buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, 2 mM Na₃VO₄) for 1 hour at 4°C. Immunoprecipitation of IGF-IR was carried out with anti-IGF-IR beta (C-20) coated protein A Sepharose CL4B (Amersham Biosciences, Sweden) from 250 µg of cell lysates. The immunoprecipitated samples were run on SDS-PAGE, under reducing condition. Following transfer to nitrocellulose membrane, levels of IGF-IR were assessed by western blot. Following an overnight incubation at 4°C, the membranes were washed 3 times with TBST and then incubated with HRP-conjugated anti-rabbit IgG diluted 1:50,000 in the same blocking buffer for 90 minutes at room temperature. After washing, blots were incubated with Super Signal (Pierce Chemicals, Rockford IL), and exposed to film.

Level of insulin receptor was determined in 15 µg of total protein run on SDS-PAGE under reducing conditions, transferred to nitrocellulose membrane, and analyzed by probing with an anti-insulin receptor antibody CT-3, diluted 1:1000 in 1% milk, 1% BSA, in TBST at 4°C overnight. The secondary, anti-mouse IgG, diluted 1:2,000 in the same blocking buffer, was applied for 90 minutes at room temperature. Blots were incubated with SuperSignal, and exposed to film.

FRET Assay

FRET assays were performed as described previously (23). Briefly, cells stably expressing a CFP-AR-YFP fusion protein (CARY) were transferred to black, clear-bottomed 96-well plates along with DHT and NDGA. The cells were fixed in 4% paraformaldehyde and read in PBS on a monochromator-based fluorescence plate reader (Safire, Tecan, Inc., NC). Each plate contained untransfected, positive, and negative controls. FRET:donor ratios were calculated following background subtraction and correction for acceptor (YFP) contribution to the FRET signal.

Real time PCR

PCR was conducted in triplicate with 20 µl reaction volumes of Taqman buffer (Applied Biosystems PCR buffer; 20% glycerol, 2.5% gelatin, 60 nM Rox as a passive reference), 5.5 mM MgCl₂, 0.5 mM each primer, 0.2 µM each deoxynucleotide triphosphate (dNTP), 200 nM probe, and 0.025 unit/µL AmpliTaq Gold (Applied Biosystems, CA) with 5 ng cDNA. A large master mix of the above-mentioned components (minus the primers, probe, and cDNA) was made for each experiment and aliquoted into individual tubes, one for each cDNA sample. cDNA was then added to the aliquoted master mix. The master mix with cDNA was aliquoted into a 384-well plate, with nine wells used for each cDNA sample. The primers and probes were mixed together and added to the master mix and cDNA in the 384-well plate. PCR was conducted on the ABI 7900HT (Applied Biosystems, CA) using the following cycle parameters: 1 cycle of 95° for 10 minutes and 40 cycles of 95° for 15 seconds, 60° for 1 minute. Analysis was carried out using the SDS software (version 2.3) supplied with the ABI 7900HT. The Ct values for each set of three reactions were averaged for all subsequent calculations. PCR primer and TaqMan probe sequences were either synthesized by Integrated DNA Technologies (Coralville, IA) or purchased from Applied Biosystems (Foster City, CA). The sequences were as follows:

H.Cyclophilin

Forward	TCTCAAATCAGAATGGGACAGGT
Reverse	TGAGAACCGTTTGTGTTGCG
Probe	5'-Fam-TTCCATTACAAGCATGATCGGGAGGGT-bhq-3'

IGF-1R:

Forward	GAGATCTTGTACATTCGCACCAAT
Reverse	TTAACTGAGAAGAGGAGTTTCGATGCT
Probe	5'-FAM- CTTTCAGTTCCTTCCATTCCCTTGGXX-BHQ1-3'

RESULTS

Androgens Stimulate the Growth of LAPC-4 Cells

The ability of the androgen, dihydrotestosterone (DHT), to stimulate the proliferation of LAPC-4 cells in culture was evaluated (Figure 1). DHT at 10 nM stimulated cell growth for up to 7 days (Figure 1a). In 3 separate experiments, at 7 days, the affect of DHT to stimulate growth was 83± 8% above control (n = 3, mean ± SEM). A major effect of DHT was observed at 0.1 nM and maximal effects were observed at 1.0 to 10 nM (Figure 1b). Two other androgens, testosterone and R1881, both at 1 nM, had similar effects (Figure 1c).

Effect of IGF-1R inhibitors on the DHT-Induced Growth

To understand the role of the IGF-1R on the DHT-induced increase in growth, we studied molecules that inhibit this receptor by unrelated mechanisms of action: NVP-AWE541 (24); and picropodophyllin (PPP) (25). The former blocks ATP binding to the receptor and the latter blocks substrate phosphorylation (24;25). Both agents completely blocked the effect of 1 nM DHT to stimulate proliferation with much smaller effects on non-androgen mediated growth (Figure 2). NVP-AWE541 was effective between 1 and 10 µM (Figure 2a), and PPP was effective between 100 and 400 nM (Figure 2b). These data support the hypothesis

therefore that DHT and the IGF-1 R may have cooperative functions in stimulating cell growth.

NDGA inhibits DHT-Induced Growth

The effect of NDGA on DHT-induced cell proliferation was first evaluated in cells grown on tissue culture plates. NDGA, at concentrations between 1 and 30 μM , inhibited androgen stimulation of growth with a smaller effect on non androgen mediated growth (Figure 3a). In 5 separate experiments, the half-maximal effect of NDGA to inhibit androgen-stimulated growth was $5 \pm 1 \mu\text{M}$ (mean $\pm$ SEM). Similar inhibition of androgen stimulation of growth was observed when the testosterone was used instead of DHT (data not shown). In addition, a similar effect of NDGA to inhibit the effect of DHT occurred when cells were grown in soft agar indicating that NDGA inhibited anchorage independent growth (Figure 3b).

Effect of NDGA on the DHT-Induced IGF-1R Autophosphorylation

In view of prior observations that NDGA rapidly inhibits ligand-induced activation of the IGF-1R in breast cancer, neuroblastoma, and other cancers, the effect of this agent on the IGF-1R in prostate cancer cells was studied. LAPC-4 cells were incubated with 1 nM DHT for either 1 or 3 days. After 1 day of incubation there was very little stimulation of this IGF-1R autophosphorylation by 10-minute incubation with IGF-1 (Figure 4a). In contrast, there was a 2-fold stimulation of this function in cells that had been incubated for 3 days with DHT (Figure 4a,b). At this time, NDGA inhibited IGF-1 induced IGF-1R autophosphorylation (Figure 4b) over a concentration range similar to that seen for inhibition of testosterone-mediated growth. In 3 separate experiments, the half-maximal effect of NDGA to inhibit IGF-1R kinase was $12 \pm 2 \mu\text{M}$ (mean $\pm$ SEM). The observation that IGF-1 activated the IGF-1R after 3 days of incubation with DHT, but not after day 1 of incubation, raised the possibility that NDGA may have had a second mechanism of action; inhibition of androgen-stimulated growth in prostate cancer cells.

DHT Increases the Expression of the IGF-1R

It has been established that the IGF-1R plays a role in the formation and growth of prostate cancer cells (3;26). Further, in prostate cancer cell lines other than LAPC-4, androgens have been shown to increase the expression and function of the IGF-1R (26;27). Accordingly, the effect of DHT on the expression of the IGF-1R in LAPC-4 cells was measured. Western blot analyses indicated that DHT markedly increased the content of the IGF-1R (Figure 5a). An effect was observed after 2 days of incubation and was near-maximal after 3 days of incubation. As with stimulation of proliferation, a significant effect of DHT was observed at 0.1 nM and maximal effects were observed at 1.0 to 10 nM (Figure 5b).

In contrast to the DHT-induced increase of the IGF-1R, there was no change in the content of the closely related insulin receptor (IR) by DHT (Figure 5c).

NDGA inhibits DHT-Induced IGF-1R content

Cells were incubated for 3 days with 1 nM DHT in the absence and presence of NDGA (Figure 5d). IGF-1R levels were increased by DHT, and this increase was progressively inhibited by increasing concentrations of NDGA from 5 to 15 μM . In 3 separate

experiments, the effects of NDGA to inhibit DHT-induced IGF-1R content was $11 \pm 2 \mu\text{M}$ (mean $\pm$ SEM) (Figure 5e).

NDGA inhibits DHT-Induced IGF-1R mRNA expression

It has been reported that androgens increase the content of the IGF-1R by increasing IGF-1R mRNA content (26). Accordingly, IGF-1R gene expression was measured by quantitative PCR and we observed that DHT increased this function near maximally at 0.1 nM (Figure 6a). The effect of NDGA on this function was then studied (Figure 6a). At 10 μM , NDGA completely inhibited the effect of DHT to increase the expression of the IGF-1R (Figure 6a).

NDGA does not inhibit DHT-Induced Changes in Androgen Receptor Conformation

Though IGF-1R transcription is stimulated by androgens, there is evidence that the IGF-1R gene itself is not directly regulated by AR, but rather it is an indirect effect involving new protein synthesis and perhaps Src and MEK1 (26). We predicted therefore that NDGA is able to inhibit androgen-induced IGF-1R expression without directly interfering with AR activation. To determine if NDGA interferes with AR activity, we employed an assay that utilizes fluorescence resonance energy transfer (FRET) to measure the conformation change induced by DHT (23). Conformation change is a proximal step in the AR activation pathway, which is likely insensitive to the secondary effects of cross-talk between AR and IGF signaling. Using a LAPC-4 cell line expressing the AR FRET reporter, it was observed that NDGA had no effect on the AR conformation change induced by DHT (Figure 6b). These results suggest that NDGA functions to inhibit IGF-1R expression at a step distal to initial AR activation.

DISCUSSION

Prior studies have indicated that NDGA can inhibit the growth of cancer cells (17;19). Although NDGA appears to have multiple effects on cancer cells, one major effect of NDGA is to directly inhibit the activation of tyrosine kinase activity of the IGF-1R. In breast cancer and neuroblastoma cells, NDGA inhibits the IGF-1R with concomitant inhibition of cell growth both *in vitro* and *in vivo* (17;19). The present studies now demonstrate that NDGA inhibits androgen-stimulated growth of LAPC-4 prostate cancer cells by several potential mechanisms. One mechanism, as observed in other cells (17;18), is direct inhibition of IGF-1R tyrosine kinase activity. Another potential mechanism of NDGA inhibition is attenuation of androgen stimulation of IGF-1R expression (26). FRET analysis of the AR suggests that the NDGA effect on AR action occurs after androgen-induced conformational changes in the AR. Our findings with NDGA and other IGF-1R inhibitors, support the hypothesis that inhibition of the IGF-1R tyrosine kinase can modulate the androgen response on prostate cancer cell proliferation, and is agreement with prior work demonstrating that the translocation of an activated AR to the nucleus for transcription is inhibited by a monoclonal antibody to the IGF-1R (28). Collectively, these data lend support to the clinical development of NDGA and other inhibitors of the IGF-1R in prostate cancer.

Prior studies from Nickerson and colleagues have demonstrated that tumor growth in xenographic mice bearing LAPC-4 tumor is associated with the expression of both the

IGF-1 receptor and its ligand, IGF-1 (5). Pandini and colleagues have reported that androgen stimulation of LNCaP cells results in an increase in expression of the IGF-1 R mRNA (26). This effect appeared to be indirect as it was blocked by inhibition of protein synthesis and inhibitors of Src and MEK1. Fan et al have also reported that androgens upregulate the IGF-1 R mRNA expression (27). Moreover, they have reported that: 1) the nuclear factor, Foxo1, inhibits AR action; and 2) IGF-1 signaling phosphorylates and inactivates Foxo1 leading to enhanced AR function. Thus, they propose a positive feedback loop between AR signaling and IGF-1R signaling (27). The present series of experiments confirm that androgens influence the expression of the IGF-1R in LAPC-4 cells and form the basis for the effect of NDGA on androgen stimulated tumor growth. The combined effects of increased IGF-1R expression in response to androgen stimulation and the potential for a cooperative effect on tumor growth by the AR and IGF-1R make IGF-1R inhibition an attractive clinical strategy therefore in patients with androgen dependent prostate cancer as well as in those with castration-resistant disease.

In order to support the hypothesis that the growth-inhibitory effects of NDGA are not occurring via direct effects on the AR, we tested the effect of this agent on conformational change of the AR in response to agonists as determined by FRET assay. There appeared to be no direct antagonism by NDGA of androgen-induced conformational changes in the AR.

Clinically, targeting an alternative (or cooperative) pathway to conventional androgen signaling provides several possibilities for providing clinical benefit to patients. The first is that by attenuating androgen signaling in tumor cells without utilizing androgen deprivation therapy, it may delay or reduce the duration of androgen deprivation and its attendant toxicities of osteoporosis, increased risk of cerebrovascular accidents and myocardial infarction, hot flashes and loss of libido. The second is that targeting the IGF receptor in the setting of androgen deprivation may improve outcomes by either prolonging the sensitivity of the tumor to the androgen deprivation therapy, increasing the proportion of cells within a tumor compartment that undergo apoptosis in response to a therapy, or attenuate one of the signals that is implicated in the emergence of castration resistant therapy. Finally, such therapies may be utilized as secondary therapies in combination with secondary androgen deprivation treatments such as adrenal androgen inhibitors or as monotherapy. Preliminary clinical evaluation of NDGA in patients with both androgen-dependent and androgen-independent prostate cancer has been performed, and has demonstrated reasonable safety and early evidence of clinical effects (29). Notably, as might be predicted by the present series of *in vitro* experiments, modest attenuating effects on the rate of rise of PSA as well as modest declines in PSA were observed occurred in patients with non-castrate levels of testosterone and a rising PSA as their only manifestation of disease (29).

In conclusion, NDGA, a small molecule with inhibitory effects on the IGF-1R, attenuates the growth stimulating effects of androgens in cultured prostate cancer cells. These effects do not occur through direct antagonism of the androgen receptor. These studies therefore support the clinical development of this agent as well as other inhibitors of the IGF-1R as alternative means of reducing androgen receptor signaling in patients with prostate cancer.

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Figure 1.

Cells were incubated in steroid-free media for 3 days and then plated in tissue culture plates with DHT and other androgens for up to 7 days. At appropriate times, cells were solubilized and proliferation measured. **1a.** The effect of duration of incubation with 10 nM DHT on cell proliferation. **1b.** The effect of increasing concentrations of DHT on cell proliferation measured after 7 days. **1c.** A comparison of the effects of DHT, testosterone (T) and the synthetic androgen, R1881, all at 1 nM on cell proliferation measured after 7 days. Each value is the mean $\pm$ SD for triplicate determinations. In 1a and 2b cell proliferation was measured by BCA the method, and in 1c by the CyQuant method. In all further studies, to measure proliferation, the CyQuant method was employed.

Figure 2.

Cells were incubated for 7 days with 1 nM DHT as in Figure 1 in the absence or presence of increasing concentrations of 2 inhibitors of the activation of the IGF-1R receptor; NVP-AWE541, and picropodophyllotoxin (PPP). **2a** NVP-AWE541. **2b**. PPP. Each value is the mean $\pm$ SD for triplicate determinations.

Figure 3.

Cells were incubated for 7 days with 1 nM DHT as in Figure 1 in the absence or presence of increasing concentrations of NDGA. **3a** Studies in tissue culture plates. **3b**. Studies in soft agar. Each value is the mean $\pm$ SD for triplicate determinations.

Figure 4.

4a. Cells were incubated with 1 nM DHT for either 1 or 3 days in the absence or presence of 3 nM IGF-1. **4b.** Cells were treated for 3 days with 1 nM DHT and then serum starved for 2 hours. Next, NDGA at increasing concentrations was added for 90 minutes, and then 3 nM IGF-1 added for 10 minutes. At that time the cells were solubilized and tyrosine phosphorylated IGF-1R measured by ELISA. Autophosphorylation activity was normalized per 10 μ g of cell protein. Each value is the mean $\pm$ SD for triplicate determinations.

Figure 5.

Cells were plated with DHT for up to 7 days. At appropriate times cells were solubilized and the IGF-1R content measured by western blots. **5a.** DHT stimulation of IGF-1R expression.

5b. Densitometric scan of the gel in 5a at 3 days. **5c.** Absence of DHT stimulation of IR expression. **5d.** Dose response of NDGA inhibition of 1 nM DHT stimulation of IGF-1R expression at 3 days. **5e.** Densitometric scan of the gel in 5d.

Figure 6.
6a. Cells were either plated with 0.1 or 1 nM DHT for 3 days, plus 10 μ M NDGA. At appropriate times cells were solubilized and the IGF-1R mRNA content measured by quantitative PCR. **6b.** Androgen receptor conformational changes measured by FRET.