Differential effects of prostate cancer therapeutics on neuroendocrine transdifferentiation

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Abstract
Androgen ablation therapy is widely used for the treatment of advanced prostate cancer. However, the effectiveness of this intervention strategy is generally short-lived as the disease ultimately progresses to a hormone-refractory state. In recent years, it has become clear that even in antiandrogen-resistant cancers the androgen receptor (AR) signaling axis is intact and is required for prostate cancer growth. Thus, there is a heightened interest in developing small molecules that function in part by down-regulating AR expression in tumors. Paradoxically, AR expression has been shown to be important in preventing the transdifferentiation of epithelial prostate cancer cells toward a neuroendocrine phenotype associated with tumor progression. Consequently, we have evaluated the relative effect of prostate cancer therapeutics that function in part by depleting AR levels on neuroendocrine differentiation in established cellular models of prostate cancer. These studies reveal that although histone deacetylase inhibitors can down-regulate AR expression they increase the expression of neuroendocrine markers and alter cellular morphology. Inhibition of AR signaling using classic AR antagonists or small interfering RNA–mediated AR ablation induces incomplete neuroendocrine differentiation. Importantly, the Hsp90 inhibitor geldanamycin effectively down-regulates AR expression while having no effect on neuroendocrine differentiation. Taken together, these data show that the phenotypic responses to pharmacologic agents used in the clinic to prevent the progression of prostate cancer are not equivalent, a finding of significant therapeutic importance. [Mol Cancer Ther 2008;7(3):659–69]

Introduction
Prostate cancer is the most frequently diagnosed malignancy in American men, second only to lung cancer as a cause of cancer death (1–3). In general, early-stage cancer that is confined to the prostate is treated effectively by surgery (that is, radical prostatectomy), while androgen ablation therapies remain the standard of care for the treatment of late-stage disease. Currently, two major approaches are used to block androgen signaling in prostate cancers: (a) chemical castration using drugs that inhibit the hypothalamic-pituitary axis and hence decrease androgen production and (b) antiandrogens, such as Casodex, that competitively inhibit androgen binding and recruit corepressors to the androgen receptor (AR). Although these approaches are initially effective, reducing both prostate size and prostate-specific antigen (PSA) levels, they invariably fail within 1 to 2 years as the tumors progress to a hormone-refractory state (2, 4).

Until recently, it was considered that prostate cancers that progressed on androgen ablation therapy would be resistant to agents that modulated the AR signaling axis. However, it has now become clear that, despite the depletion of androgenic ligands, AR and its signaling axis remain involved in the survival and growth of prostate cancer cells (4). These continued actions of AR can occur as a consequence of (a) changes in the local production of androgenic ligands; (b) overexpression of AR through gene amplification or altered mRNA expression, making it hypersensitive to low levels of androgens and/or partial agonists; (c) changes in the expression or activity of AR coregulators; (d) mutations in the receptor that either promote ligand-independent signaling or recognize atypical hormones as agonists; and (e) inappropriate activation of signal transduction pathways that interface with and activate AR in the absence of a canonical ligand (5–7). Therefore, there has been a heightened level of interest in developing therapies that function in part by down-regulating AR expression in tumors.

The epithelial cells within the prostate can be classified as basal, secretory, or neuroendocrine based on their location, morphology, and the expression of specific marker proteins (2). Although most prostatic tumors arise from secretory epithelial cells (adenocarcinomas), it has recently become apparent that neuroendocrine cells promote the progression of prostate cancer and facilitate the emergence of an androgen-independent state. Neuroendocrine cells are postmitotic and are located within the epithelial structures of the prostatic gland where they exert effects on the regulation of growth, function (e.g., secretion), and differentiation of the prostate. These cells can be identified by their expression of certain hallmark neural-specific proteins such as β-tubulin III and synaptophysin (8, 9). Importantly, these cells can secrete neuropeptides such as neotensin...
and parathyroid hormone-related peptide that function in a paracrine manner as growth factors for surrounding epithelial cells (10–12). In patient samples, proliferating epithelial cells have been found to preferentially reside close to neuroendocrine cells, highlighting the importance of localized mitogenic signaling mediated by neuroendocrine cells (13, 14). Additional studies done in vitro have shown that the conditioned medium from neuroendocrine cells can promote LNCaP and PC-3 cell growth (11, 15). Recently, it has been shown that neuroendocrine cells implanted into one flank of a castrated mouse supported the growth of LNCaP cells implanted into the other flank (16, 17). Furthermore, the transgenic adenocarcinoma of the mouse prostate model of prostate cancer shows a marked increase in prostate neuroendocrine pathology with disease progression (18). In addition, some have suggested that neuroendocrine cells may also play a role in angiogenesis by secreting factors such as vascular endothelial growth factor (19, 20).

Several different signals have been shown to induce neuroendocrine transdifferentiation of prostate epithelial cells including CAMP, interleukin-6, and serum starvation (21–24). In addition, it has been shown by several laboratories that AR represses neuroendocrine transdifferentiation (12, 25–27). Given these observations and the role of neuroendocrine cells in the progression of prostate cancers, it is important to determine how different therapeutic agents with complex actions that in part alter AR levels impact the differentiation status of cells. We believe that this information will provide additional criteria to assess the efficacy of new drugs currently undergoing clinical evaluation.

Materials and Methods

Cell Culture and Chemicals

The human prostate cancer cell lines LNCaP, PC-3, and DU145 were obtained from the American Type Culture Collection, whereas Dr. Charles Sawyers (University of California) provided the human prostate cancer cell line LAPC4. LNCaP, PC-3, or DU145 cells were cultured in RPMI 1640, Ham’s F-12K, or MEM (Eagle), respectively, supplemented with 8% fetal bovine serum (Sigma), nonessential amino acids, and sodium pyruvate (Invitrogen). LAPC4 cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 15% fetal bovine serum, nonessential amino acids, sodium pyruvate, and 1 mmol/L R1881 (Perkin-Elmer). Casodex (bicalutamide) was a gift from Dr. Philip Turnbull (GlaxoSmithKline). Geldanamycin was kindly provided by Dr. Tso-Pang Yao (Amgen). Methoxyacetic acid (MAA), valproic acid (VPA) sodium salt, sodium butyrate, and trichostatin A (TSA) were purchased from Sigma. Suberoylanilide hydroxamic acid (SAHA) was provided by Dr. William Zuercher (GlaxoSmithKline).

Western Blot Analysis

Cells were plated in 100-mm cell culture dishes at 75% confluency for 3 days in normal growth medium without additional hormones. Cells were then transfected with small interfering RNA (siRNA) or treated for various time points. Cells were harvested in lysis buffer [50 mmol/L Tris (pH 8.0), 200 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1% Triton X-100, 1 mmol/L EGTA, 10% glycerol, 50 mmol/L NaF, 2 mmol/L Na₃VO₄, and protease inhibitors] while rotating at 4°C for 30 min. Protein concentration in whole-cell extracts cleared by centrifugation was determined using the Bio-Rad protein assay. Protein (50 µg) was added to 10 µL Laemml sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue] and then boiled for 5 min. Proteins were resolved using SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with PBS-Tween (0.05%)-5% nonfat milk solution for 1 h. The membrane was subsequently incubated with antibodies to acetyl-H3 (1:2,000 dilution; Upstate), AR (AR441; 1:1,000 dilution; Dr. Dean Edwards, Baylor College of Medicine), β-tubulin III (TU1; 1:2,000 dilution; Covance), synaptophysin (SP17; 1:1,000 dilution; Covance), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; V-18; 1:1,000 dilution; Santa Cruz Biotechnology) overnight at 4°C. The next day, blots were washed in PBS-Tween (0.1%) and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (1:5,000 dilution; Bio-Rad) for 60 min at room temperature. Following three washes with PBS-Tween solution and one wash with PBS alone, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham Pharmacia Biotech) and recorded by fluorography on Hyperfilm (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Results shown are representative blots. For each sample, protein levels were determined by densitometry scanning of bands using the Image J software (NIH) and normalizing to GAPDH levels.

Real-time PCR Analysis

Cells were seeded at 500,000 per well in six-well plates and maintained for 3 days in phenol red–free medium containing charcoal-stripped fetal bovine serum (Hyclone). Cells were then treated overnight as indicated. In experiments involving cotreatments, compounds were added simultaneously. The following day, RNA was harvested using the Aurum Total RNA Mini Kit (Bio-Rad). RNA (1 µg) was then used to make cDNA using an iScript cDNA Synthesis Kit (Bio-Rad). Subsequently, quantitative real-time PCR was done using SYBR Green Supermix (Bio-Rad) as described previously (28). Results are normalized to the housekeeping control gene 36B4 and expressed as fold induction over vehicle-treated cells ± SE. Each sample was analyzed in triplicate. The sequences of the primers used are as follows: 36B4 forward GGACATGT- TTGCTGGCAATAA and reverse GGGCCCGAGAC- AGTGT; AR forward GAATTCTTGATGATAAGGC- AGCA and reverse CGAAGTCTCACAGAAGTATTGG; β-tubulin III forward GCCCTCTTCTACACGACTGCT- G and reverse CCCACTGTGACAAAGATTGA; synapto- taxisin forward GGCTACACGGCTCTATGG and reverse TGGGCTTACGTACCAGACT, PSA forward...
CCTCCTGAAAGTACCGATTC and reverse GAGTCC-ACACACTGAAGTT, and FKBP51 forward CGGAGAAC-CAAACGGAAGG and reverse CTTCGGCCCACAGTG-AATGC.

**Light Microscopy**

LNCaP or LAPC4 cells were plated at 500,000 per well in six-well plates in regular medium. Cells were then treated for 72 h as indicated in the figure legend. Light microscope photographs of cells were taken using a Nikon Coolpix 990 digital camera on an Olympus CK2 inverted microscope.

**Immunofluorescence Microscopy**

LNCaP cells were plated at 5,000 per well in eight-well poly-d-lysine-coated culture slides (BD Biosciences). Cells were then treated with water (vehicle) or 5 mmol/L VPA for 72 h after which they were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Fixed cells were washed and then permeabilized with PBS containing 0.2% Triton X-100 for 5 min. Permeabilized cells were washed, blocked for 1 h at room temperature in PBS containing 10% goat serum and 0.05% Tween, and then incubated with a 1:500 dilution of anti-β-tubulin III (TUJ1; Covance) overnight at 4°C. The following day, cells were washed in PBS-Tween (0.05%), incubated with a 1:200 dilution of Alexa Fluor 568-conjugated goat anti-mouse antibody (Invitrogen), and counterstained with 0.5 ng/mL 4,6-diamidino-2-phenylindole. Following three washes with PBS-Tween solution and two washes with PBS alone, cells were imaged on a Zeiss Axio Imager upright wide-field fluorescence microscope. Images were captured on a Hamamatsu ORCA ER CCD camera, and the system was controlled by MetaMorph.

**Detection of Cleaved Poly(ADP-Ribose) Polymerase Using Flow Cytometry**

Cells were plated in 100-mm cell culture dishes at 75% confluency for 3 days in normal growth medium followed by treatment with water (vehicle) or 5 mmol/L VPA for 72 h. Cells were then collected by pooling cell culture supernatant (floating cells) with trypsinized adherent cells and centrifuging for 5 min at 3000 × g. The resultant cell pellet was washed once with PBS and then resuspended in IC Fixation Buffer (1 × 10⁶ cells/mL; eBioscience) for 20 min at 4°C. Cells were then washed twice with PBS and resuspended at 500,000 per 100 µL in 1× Permeabilization Buffer (eBioscience) supplemented with 2% fetal bovine serum for 10 min. Aliquots (100 µL) of cells were then incubated with FITC-conjugated poly (ADP-ribose) polymerase cleavage-specific antibody or FITC-conjugated isotype control according to the manufacturer’s recommendations (Invitrogen). Cells were then washed twice in 1× Permeabilization Buffer and once in PBS and finally resuspended in 350 µL PBS. Analysis was conducted on a FACSVantage (BD Biosciences) at the Duke Cancer Center FACS Facility.

**siRNA Transfection of Human Prostate Cell Lines**

Validated Stealth siRNA directed against a scramble sequence or three different regions of AR were obtained from Invitrogen. Cells were transected for 3 days with 50 nmol/L siRNA final concentration using DharmaFECT-1 transfection reagent (Dharmacon) according to the manufacturer’s recommendations.

**Cell Proliferation Assay**

LNCaP or LAPC4 cells were seeded at 5,000 per well in 96-well plates in charcoal-stripped fetal bovine serum–containing medium and maintained for 3 days. Cells were then treated three times as indicated in the figure legend on days 4, 6, and 8. On day 11, cell proliferation was assayed to give a resultant total 7-day treatment assay. Proliferation was assayed by measuring the cellular DNA content using the FluoReporter Blue Fluorometric dsDNA Quantitation Kit (Invitrogen) as per the manufacturer’s protocol. Wells with no cells served as blanks. The fluorescence intensity was measured (excision, 360 nm; emission, 460 nm) with a fluorescence microplate reader (Fusion Universal Microplate Analyzer; Perkin-Elmer) and the fluorescence intensity of blank wells was subtracted as background. The fluorescence intensity of each sample was normalized to vehicle-treated control.

**Results**

**Treatment of Prostate Cancer Cells with Histone Deacetylase Inhibitors Induces a Neuroendocrine Phenotype**

Previous studies have shown that inhibition of AR signaling leads to a transdifferentiation toward a neuroendocrine phenotype (11, 12, 25, 26). Given that histone deacetylase (HDAC) inhibitors such as TSA have been shown to inhibit AR-mediated signaling in part through the down-regulation of AR levels (29), we hypothesized that this class of drugs could alter neuroendocrine transdifferentiation. The transition toward a neuroendocrine phenotype was evaluated by measuring the expression of the validated markers β-tubulin III and synaptophysin. The former was chosen because its expression is considered one of the earliest events that occurs in neuronally differentiating cells (9, 30, 31). Synaptophysin is normally expressed in synaptic vesicles, and although its function is largely unknown, it is a hallmark of more mature neuroendocrine cells (8). Hence, β-tubulin III is a sensitive marker for early neuroendocrine transdifferentiation, whereas synaptophysin serves as a marker for more differentiated cells. Both of the hydroxamates SAHA and TSA and the short-chain fatty acid (SCFA) VPA increased histone acetylation (Supplementary Fig. S1), decreased AR levels, and increased both the early neuroendocrine marker β-tubulin III and the mature marker synaptophysin in...
LNCaP and LAPC4 cells (Fig. 1A). Although it is known that acetylation of AR itself affects its transcriptional activity (32), our data indicate that HDAC inhibitors (HDACI) regulate AR also at the level of mRNA expression (Fig. 1B).

The dramatic effects of VPA on neuroendocrine marker expression correlated with morphologic changes typical of neuroendocrine transdifferentiation in both LNCaP and LAPC4 prostate cancer cells (that is, cell body compaction

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**Figure 1.** HDACI treatment leads to down-regulation of AR and neuroendocrine transdifferentiation of LNCaP or LAPC4 cells. **A,** LNCaP or LAPC4 cells were treated for 24 or 72 h with vehicle (-) or increasing concentrations of SAHA (0.2, 2, or 5 mmol/L), TSA (0.01, 0.1, or 1 mmol/L), or VPA (0.2, 2, or 5 mmol/L). Protein extracts were then subjected to Western blot (top) and probed for acetyl-H3, AR, β-tubulin III (β-tub III), synaptophysin (syn), or GAPDH (loading control). Bottom four graphs, densitometry results of AR, β-tubulin III, and synaptophysin Western blots normalized to GAPDH levels. Results are expressed as % AR levels of vehicle-treated cells ± SE (AR) or fold induction over vehicle-treated cells ± SE (β-tub III and synaptophysin; n = 4). * * , P < 0.05, significant changes from vehicle-treated cells.

**B,** LNCaP or LAPC4 cells were treated for 16 h with vehicle or increasing concentrations of HDACIs as in **A**. RNA was then extracted from treated cells and used for real-time PCR analysis of AR, β-tubulin III, or synaptophysin expression and normalized to 36B4. Results are expressed as fold induction over vehicle-treated cells ± SE (β-tubulin III and synaptophysin; n = 4). * , P < 0.05, significant changes from vehicle-treated cells.

**C,** LNCaP or LAPC4 cells untreated or treated for 72 h with 5 mmol/L VPA were photographed for morphologic changes as described in Materials and Methods (n = 4).
of the neuroendocrine phenotype, we next addressed differentiation toward a neuroendocrine phenotype. prostate cells that survive treatment with HDACIs may flow cytometry (Fig. 2B). These results suggest that those specific cleavage of poly(ADP-ribose) polymerase using apoptosis as determined by staining for the apoptosis-LNCaP cells treated identically with VPA underwent apoptosis in LNCaP cells after 72 h (Fig. 2A). Interestingly, only 21% of h neuroendocrine marker copy revealed a uniform increase in staining for the treated prostate cancer cells. Immunofluorescence microscopy showed that HDACIs affect AR down-regulation by different mechanisms. Taken together, these data indicate that HDACs regulate AR levels and neuroendocrine differentiation at both mRNA and protein levels.

The ability of HDACIs to transdifferentiate prostate cancer cells suggests that this class of drugs, while inducing apoptosis in some cells, may promote the generation of a subpopulation of potentially harmful neuroendocrine cells. We therefore decided to determine the relationship between transdifferentiation and apoptosis in HDACI-treated prostate cancer cells. Immunofluorescence microscopy revealed a uniform increase in staining for the neuroendocrine marker β-tubulin III in all VPA-treated LCaP cells after 72 h (Fig. 2A). Interestingly, only 21% of LCaP cells treated identically with VPA underwent apoptosis as determined by staining for the apoptosis-specific cleavage of poly(ADP-ribose) polymerase using flow cytometry (Fig. 2B). These results suggest that those prostate cells that survive treatment with HDACIs may differentiate toward a neuroendocrine phenotype.

SCFAs Promote Robust Neuroendocrine Transdifferentiation

Given that the SCFA VPA was the most effective inducer of the neuroendocrine phenotype, we next addressed whether other SCFAs would function in a similar manner. VPA, MAA, and sodium butyrate, three SCFAs we have shown previously to be effective HDACIs (37), all increased histone acetylation (Supplementary Fig. S3) and β-tubulin III and synaptophysin expression in both LCaP and LAPC4 cells (Fig. 3). As observed in VPA-treated cells, the effects of MAA and sodium butyrate also persisted 72 h after treatment. Surprisingly, whereas VPA and sodium butyrate decreased AR levels relative to their HDACI activity in both cell lines, MAA only decreased AR (62% decrease at 24 h in LCaP compared with no decrease in LAPC4) despite showing both HDAC inhibition and neuroendocrine differentiating effects in both cell lines. This discordance between decreases in AR levels and neuroendocrine differentiation suggests that these processes may be mechanistically distinct.

HDACIs Can Promote Neuroendocrine Features Independent of AR Activity

We next explored the hypothesis that HDAC-mediated neuroendocrine differentiation does not require AR modulation. Analysis of the classic AR-regulated genes PSA and FKBP51 by real-time PCR showed that whereas the HDACs VPA and MAA and the antiandrogen Casodex all inhibit androgen-mediated expression, VPA is a stronger...
inhibitor of AR activity than either MAA or Casodex (Fig. 4A). In fact, 10 μmol/L Casodex inhibited R1881-induced FKBP51 expression by only 15% in LNCaP cells. The substantial repression of AR activity by VPA extended to the inhibition of R1881-mediated prostate cell proliferation with 2 mmol/L VPA suppressing R1881-mediated cell proliferation to basal growth levels (Fig. 4B). Of note, a lower concentration of R1881 (100 pmol/L) was used for the proliferation assays because LNCaP cells show a biphasic growth curve with peak proliferation at 100 pmol/L (40). Thus, predictably, 10 μmol/L Casodex suppressed R1881-stimulated cell growth (72% decrease) more effectively than it did gene expression (PSA, 40% decrease; FKBP51, 15% decrease) in LNCaP cells.

Despite the potent anti-AR activity of HDACIs, there was a disconnect between AR activity and differentiation status as shown by the greater induction by VPA of β-tubulin III expression (5.4-fold increase) than the complete abrogation of AR by siRNA (2.5- to 2.8-fold β-tubulin III increase; Fig. 4C). Finally, using two AR-negative cell lines, PC-3 and DU145, we show that HDACIs can significantly increase the expression of neuroendocrine markers in the absence of AR (Fig. 4D). However, VPA-induced morphologic changes in these AR-negative cell lines were less dramatic compared with those seen in LNCaP and LAPC4 cells (data not shown). In conclusion, although HDACIs may increase transdifferentiation in part through the down-regulation of AR signaling, they can also promote the neuroendocrine phenotype by mechanisms that are independent of AR status. At this time, however, we do not know the exact role that down-regulation of AR plays in HDACI-mediated neuroendocrine differentiation. Indeed, we cannot exclude the possibility that HDACIs may promote transdifferentiation entirely through AR-independent mechanisms.

**AR Inhibition Alone Only Facilitates Partial Neuroendocrine Differentiation**

Given the probability that HDAC-mediated neuroendocrine differentiation may occur through multiple mechanisms, we determined the effect of AR alone on transdifferentiation. To this end, we show that ablation of

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**Figure 3.** SCFAs are effective inducers of the neuroendocrine phenotype. LNCaP or LAPC4 cells were treated for 24 or 72 h with vehicle or increasing concentrations of VPA (0.2, 2, or 6 mmol/L), MAA (1, 5, or 25 mmol/L), or sodium butyrate (0.2, 2, or 5 mmol/L). Protein extracts were then subjected to Western blot (top) and probed for acetyl-H3, AR, β-tubulin III, synaptophysin, or GAPDH. Bottom four graphs, densitometry results of AR, β-tubulin III, and synaptophysin Western blots normalized to GAPDH levels. Results are expressed as % AR levels of vehicle-treated cells ± SE (AR) or fold induction over vehicle-treated cells ± SE (β-tubulin III and synaptophysin; n = 3). *, P < 0.05, significant changes from vehicle-treated cells.
Figure 4. HDACIs block AR signaling but can promote neuroendocrine transdifferentiation through AR-independent mechanisms. A, LNCaP or LAPC4 cells were cotreated for 16 h with vehicle or 10 nmol/L R1881 in the presence of vehicle (Veh) or increasing concentrations of VPA, MAA, or Casodex (Cas). RNA was then extracted from treated cells and used for real-time reverse transcription-PCR analysis of PSA or FKBP51 expression, which were normalized to 36B4. Results are expressed as fold induction over vehicle-treated cells ± SE (n = 3). *, P < 0.05, significant changes from R1881 alone-treated cells.

B, LNCaP or LAPC4 cells were cotreated for 7 d with vehicle or R1881 in the presence of vehicle or increasing concentrations of VPA, MAA, or Casodex. Cell number was assayed using a fluorometric dsDNA quantitation kit. Results are expressed as relative cell number compared with vehicle-treated cells ± SE (n = 3). *, P < 0.05, significant changes from R1881 alone-treated cells.

C, LNCaP cells were treated with either vehicle or 5 mmol/L VPA or transfected with siRNA against scramble (Con) or three different sequences targeting AR (AR-1, AR-2, and AR-3). Following 72-h treatment or transfection, equal amounts of protein extracts were subjected to Western blot analysis (top). Blots were probed for either AR or β-tubulin III. Bottom, densitometry results of β-tubulin III Western blots normalized to GAPDH levels. Results are expressed as fold induction over vehicle-treated cells ± SE (n = 3). *, P < 0.05, significant changes from vehicle-treated or control-transfected cells.

D, PC-3 or DU145 cells were treated with vehicle or 5 mmol/L VPA for 72 h. Equal amounts of protein extracts were then subjected to Western blot analysis (top). Blots were probed for either β-tubulin III or GAPDH. Results are expressed as fold induction over vehicle-treated cells ± SE (n = 3). *, P < 0.05, significant changes from vehicle-treated cells.
AR signaling using siRNA in both LNCaP and LAPC4 prostate cancer cells induces morphologic changes typical of neuroendocrine transdifferentiation (Fig. 5A). However, the morphologic changes were less dramatic than those induced by VPA treatment (compare Figs. 1C and 5A). We next compared the acquisition of neuroendocrine characteristics subsequent to the inhibition, by different modalities, of AR signaling in LNCaP and LAPC4 cells. Inhibition of AR signaling in both LNCaP and LAPC4 cells by siRNA-mediated knockdown of AR or treatment with Casodex led to significant increases in β-tubulin III expression (Fig. 5B). Despite these various approaches to down-regulate AR expression/activity, no induction of synaptophysin expression was detected (data not shown). Knockdown of AR for 72 h increased β-tubulin III levels 2.9 ± 0.1– and 1.8 ± 0.2–fold in LNCaP and LAPC4 cells, respectively, compared to treatment with Casodex for 72 h, which only increased β-tubulin III levels 2.2 ± 0.1–fold in LNCaP cells and 1.1 ± 0.2–fold in LAPC4 cells (Fig. 5B). Because Casodex was able to increase β-tubulin III levels without altering AR levels, it appears that a decrease in AR activity is sufficient to drive the neuroendocrine transdifferentiation. Interestingly, HDACI-induced neuroendocrine differentiation was not altered by the addition of antiandrogens (Supplementary Fig. S4). This is most likely due to the fact that HDACIs can completely block AR signaling, making the addition of a competitive AR antagonist redundant. These data together suggest that inhibition of AR activity alone leads to only a partially differentiated neuroendocrine phenotype.

Hsp90 Inhibition Blocks AR Signaling but Does Not Cause Neuroendocrine Transdifferentiation

Although AR down-regulation as a means to inhibit prostate cancer growth is a well-justified approach, the neuroendocrine differentiation associated with available strategies may have undesired consequences. This led us to examine other therapeutics that could function in part through AR down-regulation but which would have a minimal effect on neuroendocrine differentiation. We hypothesized that pharmacologic inhibitors of AR signaling possessing unique mechanistic properties from those of classic antiandrogens and HDACIs could have negligible effects on neuroendocrine differentiation. Indeed, at 1 μmol/L, a concentration that completely blocks R1881-mediated PSA expression (Fig. 6B) and decreases AR protein levels greater than 75% after 6 h of treatment, the Hsp90 inhibitor geldanamycin had no effect on β-tubulin III levels (Fig. 6A). Distinct morphologic features of neuroendocrine cells were clearly visible after treatment with VPA but not geldanamycin (Fig. 6C). The inability of Hsp90 inhibition to induce the neuroendocrine phenotype may be due to a loss of other factors that results in an override of the down-regulation of AR. Regardless, the failure of geldanamycin to promote neuroendocrine differentiation further indicates that AR is not the master regulator of neuroendocrine transdifferentiation, but rather it regulates one possible pathway. Furthermore, functional Hsp90 is required for transdifferentiation induced by AR inhibition. Collectively, these data illustrate that different AR modulators being used in clinical trials for the treatment of prostate cancer may produce significantly different cell populations within the prostate, a finding of potential clinical importance.

Figure 5. Modulation of AR alone leads to incomplete neuroendocrine differentiation. A, LNCaP and LAPC4 cells transfected for 72 h with siRNA against scramble (siControl) or AR (siAR) were photographed for morphologic changes as described in Materials and Methods (n = 3). B, LNCaP and LAPC4 cells grown in normal medium were either transfected for 72 h with siRNA against scramble or AR or treated with vehicle or 1 μmol/L Casodex for indicated times. Western blots (top) were then done using equal amounts of protein extracts and probing for AR, β-tubulin III, synaptophysin, or GAPDH expression. Bottom two graphs, densitometry results of AR and β-tubulin III Western blots normalized to GAPDH levels. Results are expressed as % AR levels of siControl-transfected cells or fold induction over siControl-transfected cells (n = 3). ND, not detected. *, P < 0.05, significant changes from siControl-transfected or vehicle-treated cells.
Discussion

Whereas early-stage prostate cancers are often treated effectively by surgery alone, late-stage cancers are treated with antiandrogens and drugs that lower serum testosterone levels. Ultimately, however, these cancers progress and become resistant to these types of treatments (2, 4). Because of this phenomenon, a major area of recent drug development in this field is directed toward the identification of therapeutics that can inhibit the growth of hormone-refractory cancers (6). However, as we show here, some of the different approaches developed thus far may produce very different cell populations within the prostate.

Interestingly, despite exhibiting a hormone-refractory phenotype, advanced prostate cancers still show sustained or even increased AR signaling, an activity that appears to support disease progression (5–7). However, reports that both androgens and more directly a recent report by Wright et al. showing AR suppresses the transdifferentiation of prostate epithelial cells to a neuroendocrine phenotype prompted us to determine if different therapeutic approaches directed toward AR inhibition had similar phenotypic consequences in cells (12, 25–27, 41). This is critically important to understand, given the increasing role being ascribed to neuroendocrine cells in the development of prostate cancer and, in particular, in hormone-refractory disease (2, 42).

In this study, we show that HDACIs can promote neuroendocrine differentiation in an AR-independent manner. This raises the question of how this occurs. Work by Zhang et al. shows that androgen depletion leads to transdifferentiation through increased levels of Hsp90 (12, 25–27, 41). This is critically important to understand, given the increasing role being ascribed to neuroendocrine cells in the development of prostate cancer and, in particular, in hormone-refractory disease (2, 42).

In Figure 6, we show that Hsp90 inhibition down-regulates AR signaling but does not cause neuroendocrine transdifferentiation. A, LNCaP or LAPC4 cells were treated with vehicle, 1 μmol/L geldanamycin (GA), or 5 mmol/L VPA for indicated times. Protein extracts were then subjected to Western blot (top) and probed for AR, β-tubulin III, or GAPDH. Bottom two graphs, densitometry results of AR and β-tubulin III Western blots normalized to GAPDH levels. Results are expressed as % AR levels of vehicle-treated cells (AR) or fold induction over vehicle-treated cells (β-tubulin III; n = 3). *, P < 0.05, significant changes from vehicle-treated cells. B, LNCaP cells were cotreated for 16 h with vehicle or 10 nmol/L R1881 in the presence of vehicle or increasing concentrations of VPA or geldanamycin. RNA was then extracted from treated cells and used for real-time PCR analysis of PSA expression and normalized to 36B4. Results are expressed as fold induction over vehicle-treated cells (n = 3). *, P < 0.05, significant changes from R1881 alone-treated cells. C, LNCaP cells untreated or treated for 72 h with 5 mmol/L VPA or 1 μmol/L GA were photographed for morphologic changes as described in Materials and Methods (n = 3). D, summary of AR modulator effects on neuroendocrine transdifferentiation. HDACIs promote neuroendocrine transdifferentiation, whereas other AR modulators such as Hsp90 inhibitors do not. Inhibition of AR by antiandrogens or siRNA directed toward AR promotes neuroendocrine characteristics that lie centered on a continuum of the effects seen by inhibitors of HDACs and Hsp90.
receptor-type protein-tyrosine phosphatase α and subsequent activation of extracellular signal-regulated kinase (ERK) (26). Interestingly, although the apoptotic effects of HDACIs such as VPA are well known, Hao et al showed that VPA promotes neuronal growth through ERK signaling (43). Additionally, we have shown previously that the SCFA MAA, which we showed in this article induces transdifferentiation (Fig. 3), potentiates ERK phosphorylation/activation (37). Thus, it is possible that in prostate epithelial cells HDACIs may induce neural transdifferentiation through increased ERK signaling. In support of this Pinski et al. recently determined that genistein-induced neuroendocrine differentiation is also associated with elevated ERK activity (23).

The exact role of neuroendocrine cells in prostate cancer pathology is still controversial. There is debate in the field as to whether neuroendocrine cells are most important for early prostate cancer development, progression to a hormone-refractory state, metastasis, or a combination of all three (2, 42, 44, 45). Although many reports indicate a positive correlation between the presence of a neuroendocrine population and prostate cancer, the degree of neuroendocrine positive staining does not always correlate with tumor grade (19, 42, 44). In fact, some studies have failed to find a correlation between the presence of neuroendocrine cells and grade, whereas other reports indicate that neuroendocrine cells may secrete factors, although unknown at this time, that inhibit prostate growth (2, 24, 46). Furthermore, the current methods for detection of neuroendocrine populations do not yield equivalent results. This is most likely due to differences in sampling techniques and assay protocols (2). Regardless, there is considerable support for the idea that neuroendocrine cells, although themselves often AR negative and postmitotic, promote the growth of surrounding cells in a paracrine manner. Therefore, although HDACIs block the growth of individual prostate cancer cell lines, the possibility exists in vivo that HDACIs may actually promote an antiandrogen-resistant phenotype under some circumstances.

The disparate opinions with respect to the role of neuroendocrine cells in prostate cancer may be explained by the fact that not all neuroendocrine cells exhibit the same characteristics. Different transdifferentiation stimuli appear to give rise to distinct populations of neuroendocrine cells. For example, interleukin-6 treatment of LNCaP cells leads to an irreversible neuroendocrine phenotype that inhibits growth of surrounding cells (24, 46). Conversely, cAMP or serum starvation of prostate cells gives rise to a reversible neuroendocrine phenotype that secretes neuropeptide growth factors (10, 11). To complicate matters further, human prostate cancers are known to be heterogeneous in nature and may very well consist of different types of neuroendocrine cells distributed within the same prostate (2, 47). However, the data that exist at the current time support the notion that AR inhibition in prostate epithelial cells gives rise to a growth factor–secreting neuroendocrine cell (11, 12). Certainly, more studies are needed to better define the nature of the tumor cell populations after various treatments.

Normally, neural-specific genes are either silenced or expressed at low levels in epithelial cells. The pleiotropic effects of HDACIs will most likely lead to changes in the expression of several genes. However, it is the unique plasticity of prostate epithelial cells that allows them to transdifferentiate to a cell type that promotes tumor progression. This presents a potential drawback of this class of small-molecule inhibitors for the treatment of prostate cancer. It remains to be seen if HDACIs will lead to the development of drug-resistant cancers in the clinic. Many HDACIs have been shown to induce apoptosis in cancer cell lines in vitro (29, 39), but, as we have shown here (Fig. 2), there may be a balance between inducing apoptosis and creating a cell population in vivo that could promote cancer progression.

Given their pleiotropic effects, evaluating the therapeutic potential of HDACIs and Hsp90 inhibitors will ultimately be more complex and involve more than their ability to regulate AR signaling and neuroendocrine differentiation. For example, HDACIs have been shown to have differentiating effects on various types of stem cells (48, 49). The identification of prostate cancer stem cells suggests that a major benefit of HDACIs may lie within their ability to differentiate this small cell population that many believe could give rise to recurrent disease.

Taken together, these data show that treatment of prostate cancer using agents that affect AR signaling yields variable responses with respect to neuroendocrine differentiation. The effect these treatments have on differentiation depends on the class of drugs being used. A summary of this transdifferentiation continuum is shown in Fig. 6D. The increased attention to neuroendocrine cell populations in prostate cancer suggests that monitoring the effects of novel treatments on this differentiation process may be of importance when making therapeutic choices.

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References


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