Published in final edited form as:
Prostate. 2007 April 1; 67(5): 509–520. doi:10.1002/pros.20541.

The Hsp90 Inhibitor, 17-AAG, Prevents the Ligand-Independent Nuclear Localization of Androgen Receptor in Refractory Prostate Cancer Cells

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Abstract

BACKGROUND—Androgen receptor (AR) is the key molecule in androgen-refractory prostate cancer. Despite androgen ablative conditions, AR remains active and is necessary for the growth of androgen-refractory prostate cancer cells. Nuclear localization of AR is a prerequisite for its transcriptional activation. We examined AR localization in androgen-dependent and androgen-refractory prostate cancer cells.

METHODS AND RESULTS—We demonstrate increased nuclear localization of a GFP-tagged AR in the absence of hormone in androgen-refractory C4-2 cells compared to parental androgen-sensitive human prostate cancer LNCaP cells. Analysis of AR mutants impaired in ligand-binding indicates that the nuclear localization of AR in C4-2 cells is truly androgen-independent. The hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), inhibits basal PSA expression and disrupts the ligand-independent nuclear localization of AR at doses much lower than required to inhibit androgen-induced nuclear import.

CONCLUSIONS—Hsp90 is a key regulator of ligand-independent nuclear localization and activation of AR in androgen-refractory prostate cancer cells.

Keywords

androgen receptor; Hsp90; prostate cancer; androgen-independence; intracellular localization

INTRODUCTION

Intracellular trafficking is an important mechanism in the regulation of transcription factors. In order to access its target genes, a transcription factor requires localization to the nucleus. Likewise, retention of a transcription factor in the cytoplasm prevents its activity. For steroid

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hormone receptors such as the androgen receptor (AR), localization and activity are regulated by ligand. Ligand-dependent shuttling has been effectively demonstrated with a GFP-tagged AR [1,2]. In the absence of ligand, AR resides predominantly in the cytoplasm in a multi-chaperone complex [3]. In order for AR to act as a transcription factor it has to gain entry to the nucleus. Thus, a key regulatory step in the action of AR is its nuclear translocation. Steroid receptors, including AR, contain two nuclear localization signals, NL1 in the DNA-binding domain and hinge region, and NL2 in the ligand-binding domain (LBD) [4,5]. Recently, we have reported the existence of a nuclear export signal (NES) in the LBD of AR, that causes cytoplasmic localization of the receptor in the absence of ligand [6]. Upon binding hormone, the NES is repressed and AR translocates into the nucleus. The ligand-dependent trafficking and activation of AR are important features in its regulation of the growth and maintenance of androgen responsive organs such as the prostate.

Androgens are necessary for prostate development and growth. Acting through AR, androgens regulate proliferation, differentiation, and apoptosis of prostate cells [7–11]. In the course of prostate cancer progression, tumor development is initially dependent upon androgens. Androgen ablation therapy causes a temporary tumor regression, however, tumors will eventually relapse in a hormone-refractory state. In androgen-refractory prostate cancer AR remains active despite androgen ablative conditions [12,13]. The expression of several androgen-regulated genes, such as prostate specific antigen (PSA), is restored upon prostate cancer recurrence in a tumor xenograft model [14]. The mechanism by which the apparent loss of hormone dependence occurs is poorly understood, however, several models have been proposed [7]. One recent report showed that AR is overexpressed in refractory prostate cancer and suggested that AR was hypersensitive to low levels of androgens [15]. It has also been shown that AR activation can occur independent of hormone [16]. Further, changes in the intracellular trafficking of AR leading to ligand-independent nuclear import or impairment of nuclear export could occur in the progression to androgen-independence.

Heat Shock Protein 90 (hsp90) is one of the key regulators in the androgen action pathway. Hsp90 is part of a multi-chaperone complex known to stabilize steroid receptors in a conformation receptive to ligand [17,18]. Interaction with hsp90 is also considered to prevent steroid receptors from binding to DNA [19]. The ansamycin antibiotics geldanamycin and 17-Allylamino-17-demethoxygeldanamycin (17-AAG) are specific inhibitors of hsp90 that have been useful in examining the role of hsp90 in steroid receptor regulation [20–22]. The ligand-dependent nuclear translocation of AR can be inhibited by geldanamycin, suggesting a role for hsp90 in the nuclear import of AR [23]. 17-AAG induces a dose-dependent degradation of AR and several signaling proteins implicated in oncogenesis in androgen-dependent prostate cancer cells and inhibits growth of both androgen-dependent and androgen-independent xenografts [22].

Steroid receptors such as AR are typically activated by ligand, leading to a conformational change where hsps dissociate and DNA-binding activity is acquired [17,24]. Considerable evidence suggests, however, that the association between receptors and hsps is more dynamic and that the interaction has importance in the nuclear transport of the activated receptors [25–28]. While hsp90 inhibition has been shown to prevent the ligand-induced nuclear import of AR [23], it has not been reported whether hsp90 could also function in a potential ligand-independent activation pathway for AR in refractory prostate cancer.

In this report, we use C4-2 cells as a model for androgen-refractory prostate cancer. C4-2 cells were generated through multiple stages of co-culture of androgen-dependent LNCaP prostate cancer cells with human bone fibroblast MS cells in vivo in castrated male athymic mice [29]. Compared to its parental LNCaP cells, C4-2 cells are characterized by elevated PSA expression and increased anchorage-independent growth in soft agar [30]. Here we

demonstrate that the hsp90 inhibitor, 17-AAG, prevents ligand-independent nuclear localization and activation of AR in the androgen-refractory C4-2 cells. Hsp90 is an intriguing target in multiple human cancers, and our data suggest that inhibition of hsp90 can eliminate the androgen-independent activation of AR in androgen-refractory prostate cancer.

MATERIALS AND METHODS

Expression Vector Construction

The GFP-AR, GFP-LBD, and GFP-NES^{AR} constructs were generated as previously described [6]. Mutant GFP-AR constructs were generated by PCR mutagenesis to produce the following constructs: GFP-AR^{N705S}, GFP-AR^{A748T}, and GFP-AR^{R752Q}. All expression vectors were verified by sequencing analysis.

Cell Culture and Transfection

PC3 and LNCaP prostate cancer cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). C4-2 cells were purchased from UroCor (Oklahoma City, OK) and maintained in supplemented T-Media. LNCaP-C81 cells were obtained from Ming-Fong Lin (University of Nebraska) and were cultured in RPMI as described above. All cells were cultured at 37°C in the presence of 5% CO_2 in a humidified incubator. PC3 cells were transfected via FuGENE 6 (Roche). LNCaP, C81, and C4-2 cells were transfected via Lipofectamine 2000 (Invitrogen). All cells were transfected in OPTI-MEM media. Four hours after transfection the media was changed to phenol red-free RPMI with 10% double charcoal-stripped FBS, glutamine, penicillin, and streptomycin to ensure ligand-free conditions. Localization of GFP-tagged constructs was determined by fluorescence microscopy using a Nikon TE 2000U inverted microscope. Transfected cells were categorized as having nuclear localization when nuclear fluorescence was greater than cytoplasmic fluorescence. Conversely, when cytoplasmic fluorescence was greater than nuclear fluorescence, cells were labeled as having cytoplasmic localization. When the fluorescent intensities of the nucleus and cytoplasm were indistinguishable, the cells were categorized as having even distribution. Geldanamycin, 17-AAG, and radicicol (A.G. Scientific, Inc.) were used to inhibit hsp90. Dihydrotestosterone (DHT) and the synthetic androgen, mibolerone, were used as AR agonists.

To determine whether GFP-LBD could undergo nuclear export, hormone withdrawal experiments were conducted [6]. Briefly, C4-2 cells were transfected with GFP-LBD and treated with 10 nM mibolerone to induce nuclear import of GFP-LBD. One hour prior to hormone withdrawal, the protein synthesis inhibitor cycloheximide (CHX; 50 μg/ml) was added to the media. Cells were washed three times, then incubated with ligand-free media in the presence of CHX. Localization of GFP-LBD was assessed 4 hr after hormone withdrawal. Alternatively, some cells were maintained in mibolerone and CHX in parallel.

RNA Isolation and Northern Blot Analysis

Total RNA isolation from cultured LNCaP and C4-2 cells was carried out using the guanidinium/CsCl gradient method and Northern blot analysis was performed as previously described [31]. Human PSA probes were labeled by random priming in the presence of [^{32}P] d-CTP (DuPont NEN Life Science Products, Boston, MA).

Western Blot Analysis

LNCaP and C4-2 cells were grown to confluency and lysed in buffer containing 10 mM $NaPO_4$, 1% Triton-X-100, 1 mM EDTA, 150 mM NaCl, 2 mM PMSF, 1 mM Na_3VO_4, and

protease inhibitor cocktail (Sigma). Lysates were subjected to SDS–PAGE and Western blotting was conducted using antibodies against AR (F39.4.1, Biogenex) and β-actin. To determine the effect of hsp90 inhibition on AR protein levels, C4-2 cells were transfected with GFP-AR. Twenty hours after transfection, cells were treated with various doses of 17-AAG in the presence and absence of 1 nM mibolerone. Whole cell lysates were collected 24 hr after transfection. The AR antibody, F39.4.1, was used to detect both GFP-AR and endogenous AR from C4-2 cell lysates. A mouse monoclonal antibody against GAPDH (Abcam) was used as a control to demonstrate equal loading. Densitometric analysis of the blot was determined using NIH Image 1.61 software. Ratios of GFP-AR/GAPDH and AR/GAPDH were set at 1 for untreated cell lysates.

Immunoprecipitation

PC3 cells were grown in 10 cm dishes and transfected with GFP-AR expression vectors. Twenty-four hours after transfection, cells were lysed in a modified RIPA buffer (10 mM $NaPO_4$, 1% Triton-X-100, 1 mM EDTA, 0.5% sodium deoxycholate, 150 mM NaCl, 2 mM PMSF, 1 mM $NaVO_4$, and protease inhibitor cocktail (Sigma)). Cell lysates were pre-cleared, then incubated at 4°C overnight with 0.5 μl of rabbit polyclonal GFP antibody (ab290, abcam). This immunocomplex was added to 30 μl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) and incubated overnight. Immunoprecipitates were washed three times in RIPA buffer before being resuspended in loading buffer and subjected to SDS–PAGE. Western blotting was conducted using antibodies against AR (F39.4.1, Biogenex) and Hsp90 (SPA-835, Stressgen).

Immunofluorescence

C4-2 cells on glass coverslips were treated for 4 hr with 300 nM 17-AAG or DMSO, washed with cold PBS, then fixed with cold 100% methanol for 10 min. Cells were permeabilized with 0.5% Triton X-100 and incubated in a humidifying chamber with blocking solution (5% goat serum in PBS). Next, coverslips were incubated with the anti-AR antibody F39.4.1 (Biogenex) at a 1:200 dilution in blocking solution for 1 hr. After a series of PBS washes, cells were incubated with TRITC-conjugated anti-mouse IgG (Sigma) diluted 1:200. Coverslips were washed in PBS and mounted onto slides using DAKO Glycergel mounting medium. Localization was assessed by fluorescence microscopy as described above.

RESULTS

Unliganded AR Is Localized to the Nucleus in Androgen-Refractory Prostate Cancer Cells

A prerequisite for AR transcriptional activity is its nuclear localization. To determine whether the sub-cellular localization of AR was altered during the progression to androgen-independence, AR-negative PC3 prostate cancer cells, androgen-sensitive LNCaP cells, and androgen-refractory C4-2 cells were transfected with AR tagged to green fluorescent protein (GFP) and localization was assessed by fluorescence microscopy in hormone-free conditions. Notably, GFP-AR exhibits similar androgen-binding affinity as untagged AR and androgen can induce GFP-AR transactivation [1]. As previously described, the localization of GFP-AR in PC3 cells is regulated by hormone [6]. In the absence of ligand, GFP-AR was predominantly cytoplasmic in nearly all PC3 cells, with no observed cells exhibiting predominantly nuclear localization (Fig. 1A). Less than half (40%) of transfected LNCaP cells displayed predominant nuclear localization of GFP-AR. In refractory C4-2 cells, however, GFP-AR exhibited predominant nuclear localization in 64% of transfected cells, a significant increase ($P = 0.0028$) compared to LNCaP cells (Fig. 1A). This is consistent with a previous report where increased nuclear localization of endogenous AR in C4-2 cells compared to the parental LNCaP cell line was observed by immunocytochemistry [32]. Another androgen-refractory LNCaP subline, C81 [33], also displayed nuclear localization of GFP-AR in the absence of hormone, similar

to C4-2 cells (data not shown). Treatment with 1 nM mibolerone induced complete nuclear localization of GFP-AR in PC3, LNCaP, and C4-2 cells. The hormone-induced nuclear localization in C4-2 cells appears more defined than the ligand-independent nuclear localization (Fig. 1B). This suggests that even though most GFP-AR is already in the nucleus in C4-2 cells, it remains sensitive to hormone addition. No differences were observed between the cell lines when transfected with GFP alone, which was highly expressed in both the nucleus and cytoplasm (Fig. 1B), suggesting that the differences in GFP-AR localization were due to the presence of AR rather than altered localization of GFP.

AR Is Active in the Absence of Ligand and Is Further Activated by Androgens in C4-2 Cells

To determine whether AR in C4-2 cells was transcriptionally active, Northern blot analysis of the androgen-regulated gene PSA was conducted. Both the mRNA and protein levels of PSA are known to be induced by androgen in LNCaP cells [34,35]. Another study recently showed that the basal PSA expression in C4-2 cells is still AR-dependent, as siRNA directed against AR resulted in the near-complete loss of PSA protein [36]. Thus, PSA expression reflects AR activation. Increased PSA mRNA expression in the absence of androgen was observed in C4-2 cells compared to parental LNCaP cells (Fig. 1C). This confirms that AR is in the nucleus and is active in the absence of ligand in androgen-refractory C4-2 cells. LNCaP and C4-2 cells express similar levels of AR protein (Fig. 1D), suggesting that the difference in basal PSA expression is not due to overexpression of AR in C4-2 cells. Treatment with 1 nM DHT for 24 hr upregulated PSA expression in LNCaP cells, confirming androgen-regulation of PSA expression. While the basal PSA expression was high in C4-2 cells, DHT treatment was able to further increase PSA levels (Fig. 1C), demonstrating that AR in C4-2 cells remains hormone-responsive. These findings are in agreement with a previous report which characterized PSA expression in the presence and absence of hormone in LNCaP cells and its androgen-refractory sublines, including C4-2 [30].

NES^{AR} Is Active in Androgen-Refractory C4-2 Cells

An intriguing possibility is that nuclear export of AR is impaired in C4-2 cells, leading to nuclear accumulation and activation of AR in the absence of hormone. To determine whether the nuclear localization of GFP-AR in the absence of hormone in C4-2 cells was due to a deficiency in nuclear export, we tested the possibility that the C4-2 cells failed to recognize the NES of AR (NES^{AR}). NES^{AR} is a 75 amino acid region of the LBD responsible for the cytoplasmic localization of AR [6]. Cytoplasmic localization of transfected GFP-NES^{AR} was observed in both LNCaP and C4-2 cells (Fig. 2A) suggesting that the nuclear localization of AR in C4-2 cells was not due to a failure to recognize the nuclear export signal, NES^{AR}. Similarly, the entire LBD fused to GFP (GFP-LBD) localized to the cytoplasm in C4-2 cells in hormone-free media. The addition of 10 nM mibolerone induced nuclear transfer of the GFP-LBD, demonstrating that the LBD remained hormone responsive (Fig. 2B). Further, the ability of mibolerone to induce nuclear import of GFP-LBD suggests that in our “hormone-free” experimental conditions the LBD of AR is unbound to any residual ligands potentially present in the charcoal-stripped serum. CHX was added to the cells prior to and after hormone withdrawal to prevent the synthesis of new GFP-LBD protein. We have previously demonstrated that CHX does not affect the trafficking of GFP-LBD [6]. Following hormone withdrawal, GFP-LBD redistributed to the cytoplasm (Fig. 2B), indicating that the LBD can still undergo nuclear export in androgen-refractory C4-2 cells.

Localization of AR Mutants Does Not Correlate With Hormone Sensitivity

One theory for the activation of AR in androgen-independent prostate cancer proposes that AR is hypersensitive to low levels of hormone. In support of this hypothesis, a recent study showed that an intact LBD was required for the transition to androgen independence [15]. This would

seem to suggest that hormone-refractory prostate cancer is not truly androgen-independent. To address whether the nuclear localization of AR in androgen-refractory prostate cancer also requires an intact, hormone-responsive LBD, we examined localization of GFP-tagged AR constructs with mutations found in complete androgen insensitivity syndrome (CAIS), AR^{N705S}, or partial androgen-insensitivity syndrome (PAIS), AR^{R752Q}. To examine hormone-responsive localization, PC3 cells were transfected with the GFP-AR mutants and treated with low levels of androgen (0.01 nM mibolerone). PC3 cells were used in this experiment because the localization of the GFP-tagged wild-type AR is tightly regulated by androgen in this cell line [6]. Also, since PC3 cells do not express AR, the experiment is not complicated by the possibility of dimerization between the AR mutants and liganded, endogenous AR. In the absence of hormone treatment, GFP-AR and each AR mutant used in this experiment exhibited cytoplasmic localization when transfected into PC3 cells (Fig. 3A). In cells treated with 0.01 nM mibolerone, ligand-induced nuclear import was impaired in both the CAIS mutant (GFP-AR^{N705S}) and the PAIS mutant (GFP-AR^{R752Q}), however, import of the CAIS mutant GFP-AR^{N705S} was inhibited to a greater extent (Fig. 3A). This is expected considering that AR^{N705S} has less ligand-binding activity than AR^{R752Q} [15].

If the nuclear localization of AR observed in C4-2 cells is caused by ligand-binding, then mutations which confer androgen-insensitivity should disrupt this nuclear localization. However, 49% of transfected C4-2 cells displayed predominant nuclear localization of GFP-AR^{N705S} in the absence of exogenously added hormone (Fig. 3B). Interestingly, the PAIS mutant, GFP-AR^{R752Q}, displayed a dramatic decrease in nuclear localization with a corresponding increase in cytoplasmic localization compared to GFP-AR (Fig. 3B). While GFP-AR^{R752Q} is more sensitive than GFP-AR^{N705S} to low levels of hormone in PC3 cells, its localization remains cytoplasmic in 68% of transfected C4-2 cells. Conversely, GFP-AR^{N705S}, which is less sensitive to a low concentration of mibolerone, had greater nuclear localization compared to GFP-AR^{R752Q} (49% vs. 4%) in the absence of ligand in the androgen-refractory C4-2 cells. These findings suggest that the nuclear localization of AR in refractory prostate cancer cells does not require hormone-binding and may instead be regulated by other factors.

The R752Q mutation lies within a region corresponding to the fragment in rat AR that binds to hsp90 [37,38]. Mutation of another amino acid in this vicinity, A748T, was suggested to interfere with hsp complexes [39]. Despite hormone responsive localization in PC3 cells (Fig. 3A), GFP-AR^{A748T} displayed predominant cytoplasmic localization in 52% of transfected C4-2 cells in the absence of hormone (Fig. 3B). These observations suggest that the loss of nuclear localization of AR^{A748T} and AR^{R752Q} in C4-2 cells may be due to impaired interaction with hsp90 rather than impaired binding to low levels of androgen.

To determine whether these GFP-AR mutants could interact with hsp90, co-immunoprecipitation experiments were conducted. Dimerization of the GFP-AR mutants with endogenous AR could potentially recruit hsp90 in the immunoprecipitates. Therefore, whole cell lysates from AR-negative PC3 cells, transfected with the GFP-AR mutants, were used to exclude this possibility. As expected, an interaction with hsp90 was detected for both GFP-AR and GFP-AR^{N705S} (Fig. 3C). The interaction with hsp90 was weaker, but still detectable for GFP-AR^{N748T} and GFP-AR^{R752Q} (Fig. 3C). The fact that GFP-AR^{A748T} and GFP-AR^{R752Q} retained cytoplasmic localization in C4-2 cells while displaying less binding to hsp90 suggested that hsp90 may have an important role in promoting ligand-independent nuclear localization of AR in refractory prostate cancer cells.

17-AAG Inhibits the Ligand Independent Nuclear Localization of AR in C4-2 Cells

To determine whether hsp90 regulated the ligand-independent nuclear localization of AR, androgen-refractory C4-2 cells were transfected with GFP-AR and treated with the hsp90

inhibitor, 17-AAG. After 4 hr in the presence of 300 nM 17-AAG, the localization of GFP-AR had shifted from predominantly nuclear to cytoplasmic (Fig. 4A). The nuclear localization of GFP-AR in C4-2 cells was also prevented by two other hsp90 inhibitors, geldanamycin and radicicol (data not shown). To determine if the effect of hsp90 inhibition on endogenous AR in C4-2 cells was similar to its effect on transfected GFP-AR, immunofluorescence experiments were performed. In hormone free conditions, endogenous AR is present in the nucleus of C4-2 cells, however, after 17-AAG treatment a decrease in nuclear AR expression was observed (Fig. 4B). Thus, the ligand-independent nuclear localization of both endogenous AR and transfected GFP-AR in androgen-refractory C4-2 cells can be prevented when hsp90 is inhibited.

17-AAG Inhibits Both Androgen-Induced and Hormone-Independent PSA Expression

Northern blot analysis was conducted to determine the effect of 17-AAG on androgen-regulated and hormone-independent PSA expression in LNCaP and C4-2 cells. In LNCaP cells, 17-AAG inhibited both basal and androgen-induced PSA expression (Fig. 4C). Co-treatment with 17-AAG and DHT elevated PSA expression above basal levels, suggesting that some hormone-sensitivity was retained in the presence of 200 nM 17-AAG. In C4-2 cells, ligand-independent PSA expression was downregulated in cells treated with 200 nM 17-AAG compared to the untreated controls (Fig. 4C). PSA expression could be partially restored in 17-AAG treated C4-2 cells by co-treatment with 1 nM DHT (Fig. 4C), suggesting that while the hormone-independent PSA expression could be inhibited, partial androgen sensitivity could be maintained. This suggests that 17-AAG can inhibit ligand-independent activation of AR target genes while retaining the androgen-dependent AR activation pathway.

Ligand-Independent Nuclear Localization of AR in C4-2 Cells Is More Sensitive to 17-AAG Than Ligand-Induced AR Nuclear Import

In addition to its known involvement in androgen-induced nuclear import, our findings suggest that hsp90 is also involved in the nuclear localization of unliganded AR in androgen-refractory prostate cancer cells. It was unclear whether 17-AAG affected ligand-induced and ligand-independent AR localization in the same manner. 17-AAG dose response experiments were conducted to compare the effect of hsp90 inhibition on ligand-independent AR nuclear localization and mibolerone-induced nuclear import of AR. PC3 and C4-2 cells were used because localization of GFP-AR is tightly regulated by androgen in PC3 cells, while GFP-AR is predominantly nuclear in C4-2 cells and the nuclear localization can increase further upon androgen stimulation. PC3 and C4-2 cells were transfected with GFP-AR and treated with several concentrations of 17-AAG. Some cells were simultaneously co-treated with 1 nM mibolerone to induce nuclear import of GFP-AR. Almost no inhibition of mibolerone-induced nuclear import of GFP-AR was produced by 100 nM 17-AAG in either PC3 or C4-2 cells (Fig. 5A). However, in the absence of hormone, C4-2 cells treated with 100 nM 17-AAG displayed a sharp loss of nuclear localization. The percentage of cells displaying predominant nuclear localization of GFP-AR in the presence of mibolerone did not drop below 50% in PC3 or C4-2 cells unless co-treated with 17-AAG concentrations ≥ 5 μM (Fig. 5A). Conversely, ligand-independent nuclear localization of GFP-AR was almost completely inhibited by 300 nM 17-AAG. These results demonstrate that ligand-independent nuclear localization of AR in androgen-refractory C4-2 cells is more sensitive than androgen-induced AR nuclear localization to hsp90 inhibition.

17-AAG has previously been shown to downregulate AR protein levels in a dose-dependent manner [22]. Using Western blot analysis, we tested whether inhibition of GFP-AR nuclear localization by 17-AAG coincided with a decrease in AR protein levels. C4-2 cells were transfected with GFP-AR and treated with 17-AAG and/or mibolerone, as described above. Notably, lysates were collected 4 hr after treatment, the same time point when localization was

observed. Concentrations of 17-AAG (100 nM, 300 nM, and 5 μM) were tested in the presence or absence of 1 nM mibolerone. Both 100 and 300 nM 17-AAG effectively disrupted the ligand-independent nuclear localization of GFP-AR in C4-2 cells without compromising its ligand-induced nuclear import (Fig. 5A). These concentrations of 17-AAG also caused a small reduction of GFP-AR protein levels in both the presence and absence of mibolerone (Fig. 5B). This indicates that in the presence of ligand, nuclear localization of GFP-AR may persist in the midst of 17-AAG-mediated degradation. In about 50% of ligand-treated C4-2 cells, predominant nuclear localization of GFP-AR was prevented by 5 μM 17-AAG. A dramatic loss of GFP-AR protein was observed in C4-2 cells treated with 5 μM 17-AAG in both the presence and absence of mibolerone. Endogenous AR in C4-2 cells displays a relatively similar pattern of downregulation in response to the tested concentrations of 17-AAG (Fig. 5B). Mibolerone appears to increase the stability of both endogenous AR and GFP-AR in C4-2 cells in the presence or absence of 17-AAG (Fig. 5B). Thus, stabilization of AR by androgens may counteract some of the inhibitory effects of 17-AAG.

DISCUSSION

LNCaP and C4-2 cells are frequently used as respective models of androgen-sensitive and androgen-refractory prostate cancer. LNCaP cells express an AR with a point mutation at amino acid 877. This threonine to alanine mutation allows AR to be promiscuously activated by a variety of hormonal ligands [3]. AR is found both in the nucleus and cytoplasm of LNCaP cells [40]. Likewise, transfected wild-type GFP-AR localizes to both nucleus and cytoplasm, and in many cells is evenly distributed (Fig. 1A). In this regard, LNCaP cells differ from previously characterized models of AR trafficking, where GFP-AR is predominantly localized to the cytoplasm in the absence of hormone in COS1, HeLa, and PC3 cells [2,6]. However, as observed in these other cell lines, androgen treatment induces a robust nuclear localization of GFP-AR in LNCaP cells (Fig. 1B).

It has been suggested that LNCaP cells adapted to become C4-2 cells through interaction with bone stromal cells [29]. In a recent report, profiling of cluster designation cell surface markers suggests that C4-2 cells developed from a pre-existent sub-population of LNCaP cells [41]. This explanation may account for the fact that a fraction of transfected LNCaP cells displayed predominant nuclear localization of GFP-AR (Fig. 1A). In castrated male mice C4-2 cells are tumorigenic, highlighting their androgen-independent nature [29]. Subsequent studies demonstrated elevated PSA expression and secretion under hormone-free conditions in C4-2 cells compared with the parental LNCaP cell line [30,42]. Increased nuclear localization of endogenous AR was also observed [32]. Consistent with this, GFP-AR exhibited increased nuclear localization in C4-2 cells compared to LNCaP cells (Fig. 1A). Analysis of the PSA mRNA expression levels suggests that the increased nuclear localization of AR in C4-2 cells coincides with a higher basal AR transcriptional activity (Fig. 1C). Despite the predominant nuclear localization in C4-2 cells, AR remains sensitive to hormone since mibolerone can alter GFP-AR localization, producing an enhanced nuclear distribution (Fig. 1B), and can increase PSA expression (Fig. 1C). The term “androgen-refractory” is used to describe C4-2 cells based upon their ability to proliferate in the absence of androgen. These results demonstrate that while activation of AR can occur in an androgen-independent manner, C4-2 cells are not androgen-insensitive since androgens increase both nuclear localization and PSA expression in this cell line. Interestingly, the difference in ligand-independent nuclear localization of GFP-AR between LNCaP and C4-2 cells was less pronounced than the difference in expression of the AR target gene PSA, suggesting that ligand-independent nuclear localization may be only one step in triggering AR activation and other events may be necessary to activate AR, once it is in the nucleus. Thus, we cannot rule out the possibility that other factors, acting selectively in C4-2 but not LNCaP cells, increase AR transactivation to produce enhanced PSA expression.

Nuclear localization of AR in refractory prostate cancer cells requires a mechanism directing ligand-independent nuclear import and/or a means of retaining AR in the nucleus in the absence of ligand. We investigated whether the recently characterized NES^{AR} could function in androgen-refractory C4-2 cells. A loss of GFP-NES^{AR} cytoplasmic localization would have suggested that the nuclear export pathway for AR in C4-2 cells was impaired, causing AR to remain in the nucleus. GFP-NES^{AR} displayed cytoplasmic localization, suggesting that the AR nuclear export pathway remains functional in C4-2 cells (Fig. 2A). Likewise, GFP-LBD, which underwent nuclear import in the presence of ligand, was capable of nuclear export upon hormone withdrawal (Fig. 2B).

An alternative possibility is that androgen-refractory prostate cancer is not truly androgen-independent. Rather, the AR becomes hypersensitive to extremely low levels of circulating adrenal androgens. A recent study emphasized the importance of AR overexpression in the progression to androgen-refractory prostate cancer and suggested that over-expression of AR facilitated stimulation by low levels of hormone [15]. However, LNCaP and C4-2 cells express similar levels of AR protein [43] (Fig. 1D), suggesting that overexpression is not what confers ligand-independent AR activation to C4-2 cells. Further, analysis of AIS mutants showed that localization of AR mutants in C4-2 cells does not correlate with their sensitivity to ligand (Fig. 3). While hypersensitivity of AR is thought to be common in androgen-refractory prostate cancer, our data suggest that the nuclear localization of AR in androgen-refractory C4-2 cells is genuinely ligand-independent. However, it remains possible that nuclear localization of AR is insufficient to drive prostate cancer cell growth and that further activation by low levels of hormone may still be necessary.

The mutant receptor AR^{A748T} is less stable than wild-type AR and is insensitive to GA-induced degradation, suggesting that its interaction with hsp90 may be altered [39]. In our experiments, GFP-AR^{A748T} showed similar hormone-responsiveness as wild-type GFP-AR, but was not localized to the nucleus of C4-2 cells in the absence of ligand (Fig. 3). The PAIS mutation R752Q is in the near vicinity of A748T and GFP-AR^{R752Q} is also predominantly localized to the cytoplasm in C4-2 cells. Co-immunoprecipitation experiments demonstrated that both GFP-AR^{A748T} and GFP-AR^{R752Q} displayed reduced interaction with hsp90. This suggested that hsp90 may be an important mediator of AR localization in androgen-refractory prostate cancer cells. An interaction with hsp90 was still detectable in these mutants, indicating that the ability to bind to hsp90 was not sufficient to trigger ligand-independent AR nuclear localization. However, alterations in the way hsp90 binds to AR mutants may impair the ability of hsp90 to induce nuclear localization in the absence of hormone.

Mutations in the LBD can impair hsp90-binding and ligand-independent nuclear localization of AR. GFP-LBD, however, remains in the cytoplasm in the absence of ligand in C4-2 cells (Fig. 2B). Nuclear localization of GFP-LBD in C4-2 cells was induced following treatment with 10 nM DHT, confirming that the LBD is unliganded in untreated cells and that it remains sensitive to androgen-induced nuclear import. These results suggest that an intact LBD is not sufficient to elicit ligand-independent nuclear localization in C4-2 cells, and that other regions of AR are required. While the LBD can bind to hsp90, neither is sufficient to produce nuclear localization in the absence of hormone. In the context of the full-length receptor, however, an LBD without mutations which disrupt binding to hsp90 appears to be important to produce nuclear localization in the absence of androgen. Our analysis suggested that inhibiting hsp90 may affect AR localization.

We showed that the hsp90 inhibitor 17-AAG prevented the ligand-independent nuclear localization of both GFP-AR and endogenous AR in C4-2 cells. Thus, while binding to hsp90 is not sufficient to direct mutant ARs to the nucleus, hsp90 function is required for AR nuclear localization in the absence of androgen. 17-AAG dose response experiments demonstrated that

the nuclear localization of GFP-AR observed in C4-2 cells under ligand-free conditions is much more sensitive to lower doses of 17-AAG compared to the mibolerone-induced nuclear import of GFP-AR in both PC3 and C4-2 cells (Fig. 5A). At doses less than 500 nM, 17-AAG can preferentially inhibit the androgen-independent nuclear localization of AR in C4-2 cells (Fig. 5A). This suggests that the mechanism by which hsp90 influences androgen-independent nuclear localization of AR in androgen-refractory prostate cancer cells may be different from hsp90 regulation of ligand-induced AR nuclear import. Whereas 17-AAG can prevent hsp90 from mediating the ligand-induced translocation of AR, it seems that at lower doses 17-AAG can disrupt a yet uncharacterized pathway in refractory prostate cancer cells in which a ligand-independent stimulus activates AR. Since 17-AAG treatment affects a host of hsp90 client proteins, we cannot rule out the possibility that the effects on AR localization are mediated through other molecules whose activities are disrupted by 17-AAG. However, the reduced nuclear localization of AR mutants whose interactions with hsp90 are impaired suggest that the effects of 17-AAG on AR localization are mediated directly via hsp90.

Occupancy by androgen stabilizes AR [44], whereas 17-AAG promotes AR degradation [22]. The differential sensitivities of liganded and unliganded GFP-AR to 17-AAG may reflect a role for ligand in counteracting the inhibitory effects of 17-AAG. Western blot analysis confirmed that mibolerone and 17-AAG have opposing effects on the stability of AR (Fig. 5B). While 17-AAG was still able to downregulate AR, the levels of AR protein remained higher in the presence of mibolerone than in its absence. Thus, mibolerone does not completely protect AR from 17-AAG. However, the stabilizing effect of mibolerone may be sufficient to sustain nuclear localization of AR in the presence of low 17-AAG concentrations. Conversely, in the absence of hormone, 17-AAG reduces both the nuclear localization and the protein levels of AR. If clinically applicable, this would suggest that lower doses of 17-AAG can most effectively abrogate AR signaling under androgen ablative conditions.

Northern blot analysis of the PSA transcript con-firmed previous reports that AR was active in C4-2 cells in hormone-free conditions (Figs. 2 and 4C). 17-AAG inhibited this hormone-independent activity. In the presence of DHT, PSA expression was partially restored in 17-AAG treated C4-2 cells (Fig. 4C). Thus, it appears that hsp90 inhibition can target ligand-independent AR activation in refractory prostate cancer cells while retaining androgen sensitivity. The presence of androgen can oppose the effects of 17-AAG on AR stability, localization, and activity.

During the progression to androgen-refractory prostate cancer, the tightly regulated androgen signaling pathway is disrupted such that unliganded AR can localize to the nucleus and activate its target genes. Our data suggest that 17-AAG can block this ligand-independent pathway, partially restoring androgen-dependent regulation of AR in refractory prostate cancer cells, and thus potentially providing a new solution to improve the efficacy of androgen ablation therapy. By targeting hsp90, 17-AAG prevents ligand-independent nuclear localization of AR, decreases AR stability, and inhibits basal PSA expression in androgen-refractory C4-2 cells. It is currently unclear whether the shift in unliganded GFP-AR localization, from predominantly nuclear to predominantly cytoplasmic in the presence of 17-AAG, is due to decreased ligand-independent nuclear import, increased nuclear export, selective degradation of nuclear GFP-AR, or some combination of these possibilities. The exact mechanism by which hsp90 and its inhibitor influence the ligand-independent nuclear localization and activation of AR in C4-2 cells remains to be determined in future studies. Hsp90 has been identified as an attractive target in prostate cancer and hormone-refractory breast cancer because its client proteins include steroid receptors and oncogenes [22,45]. These results suggest that the hsp90 inhibitor, 17-AAG, may be particularly effective at targeting the androgen-independent activation of AR in androgen-refractory prostate cancer.

Acknowledgments

Grant sponsor: NIH; Grant numbers: R01 CA 108675, 5 R01 DK51993, 1 P50 CA90386; Grant sponsor: NCI; Grant sponsor: Robert H. Lurie Comprehensive Cancer Center (NIH training grant); Grant number: T32 CA80621.

This investigation was supported in part by NIH grants R01 CA 108675, 5 R01 DK51993, 1 P50 CA90386. A.J.S. was supported by NCI, NIH training grant T32 CA80621 from the Robert H. Lurie Comprehensive Cancer Center. J. Ai is the recipient of an AFUD grant. Special thanks to Kathy Rundell and Minh Nguyen for critical reading of the manuscript and Ming-Fong Lin for kindly providing LNCaP-C81 cells.

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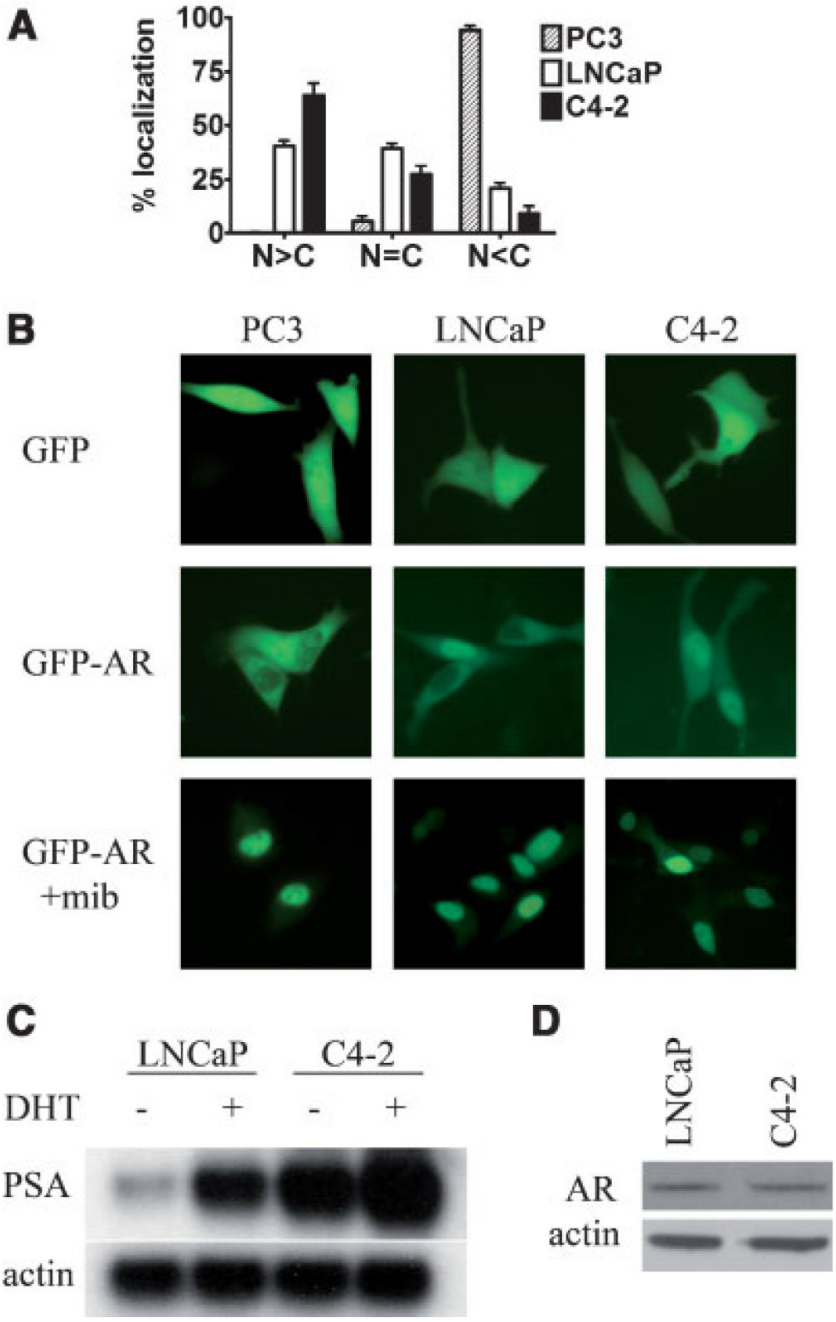

Fig. 1.

A, B: Localization of GFP-AR in androgen-dependent and androgen-refractory prostate cancer cells. PC3, LNCaP, and C4-2 cells were transfected with GFP-AR and localization was assessed in ligand-free conditions or in the presence of 1nM mibolerone by fluorescence microscopy within 24 hr of transfection. The results are from five transfections for each cell line. At least 50 cells were counted after each transfection. Error bars represent ± SEM. A *P*-value < 0.05 was generated using an unpaired *t*-test in GraphPad Prism (GraphPad Software, Inc.). **C**: AR is active in the absence of hormone in androgen-refractory C4-2 cells. LNCaP and C4-2 cells were treated with or without mibolerone for 24 hr. Northern blot analysis determined PSA mRNA expression in the presence and absence of hormone. β-actin mRNA

is shown as a loading control. **D:** AR protein levels in LNCaP and C4-2 cells. β-actin protein is shown as a loading control.

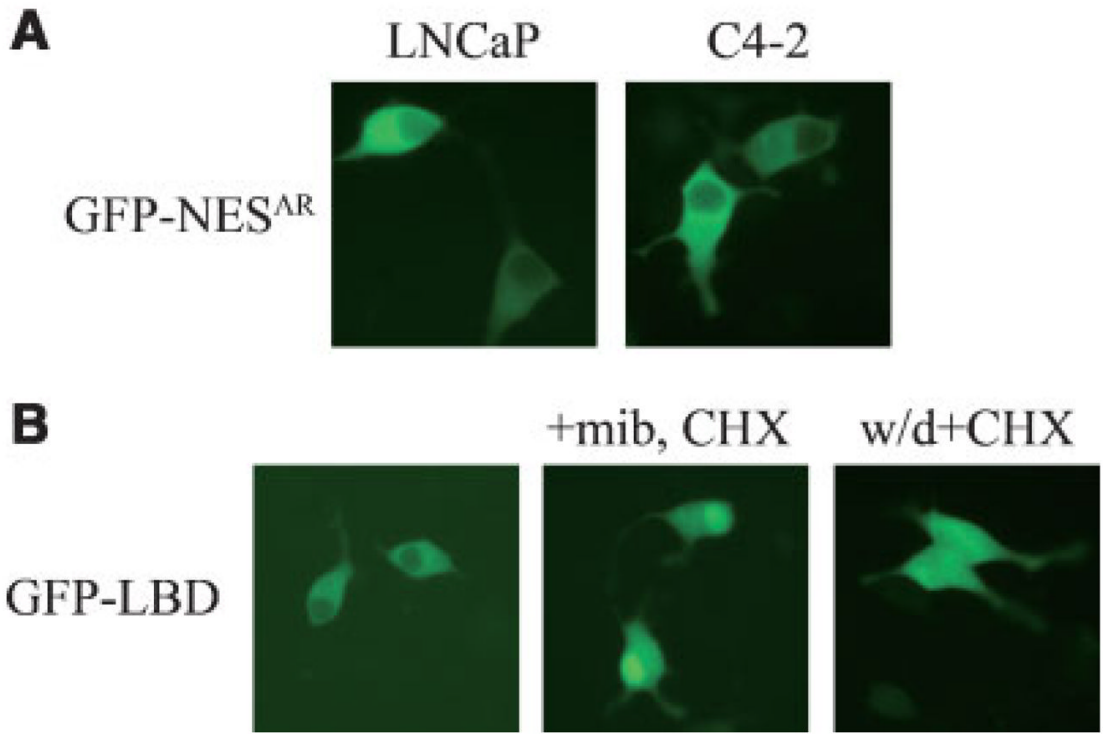

Fig. 2.

A: NES^{AR} is localized to the cytoplasm in androgen-sensitive and androgen-refractory prostate cancer cells. LNCaP and C4-2 cells were transfected with GFP-NES^{AR} and localization was assessed in ligand-free conditions by fluorescence microscopy. **B:** The localization of GFP-LBD is regulated by androgen in C4-2 cells. Localization of transfected GFP-LBD was assessed in the absence, presence, and following withdrawal of 10 nM mibolerone. Cycloheximide (CHX) was included as a control to prevent de novo synthesis of GFP-LBD protein.

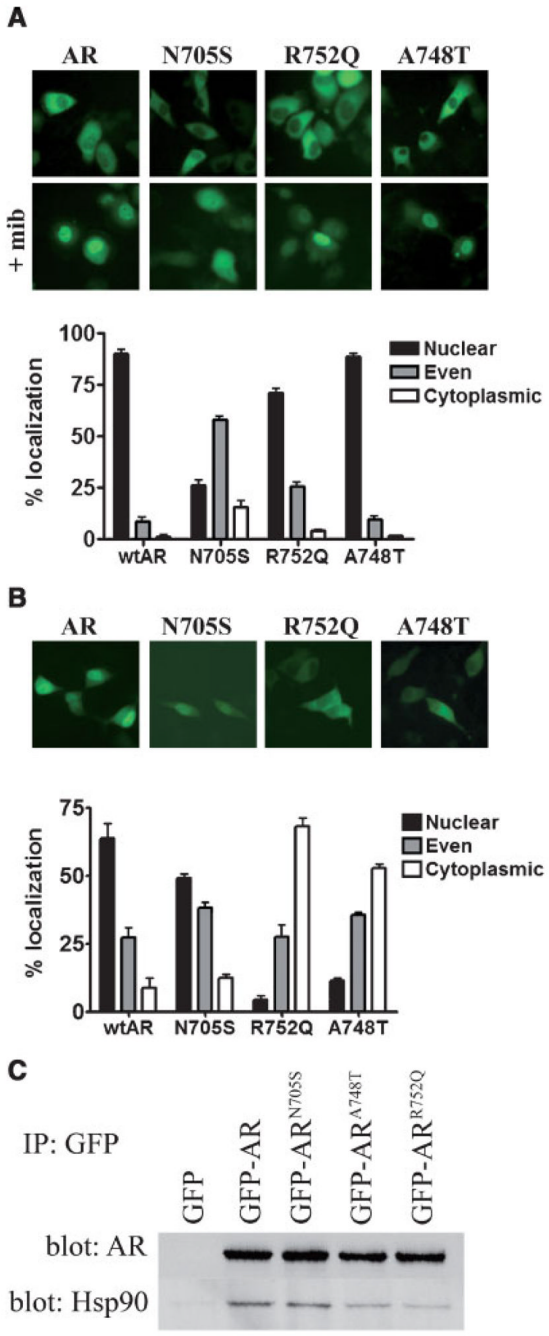

Fig. 3.

Localization of AR mutants in the absence of exogenous androgen is associated with hsp90-binding but not hormone-sensitivity. The GFP-tagged androgen receptor mutant constructs AR^{N705S}, AR^{R752Q}, AR^{A748T}, or wild-type AR were transfected into PC3 and C4-2cells. **A:** PC3 cells were treated with 0.01 nM mibolerone to determine the responsiveness of the AR mutants to low doses of androgen. **B:** In C4-2 cells intracellular localization of the AR mutants was assessed in the absence of hormone. Error bars represent ± SEM. **C:** Transfected PC3 cells were lysed and AR mutants were immunoprecipitated with a GFP antibody. Western blotting was used to detect an interaction between the immmunoprecipitated AR and hsp90.

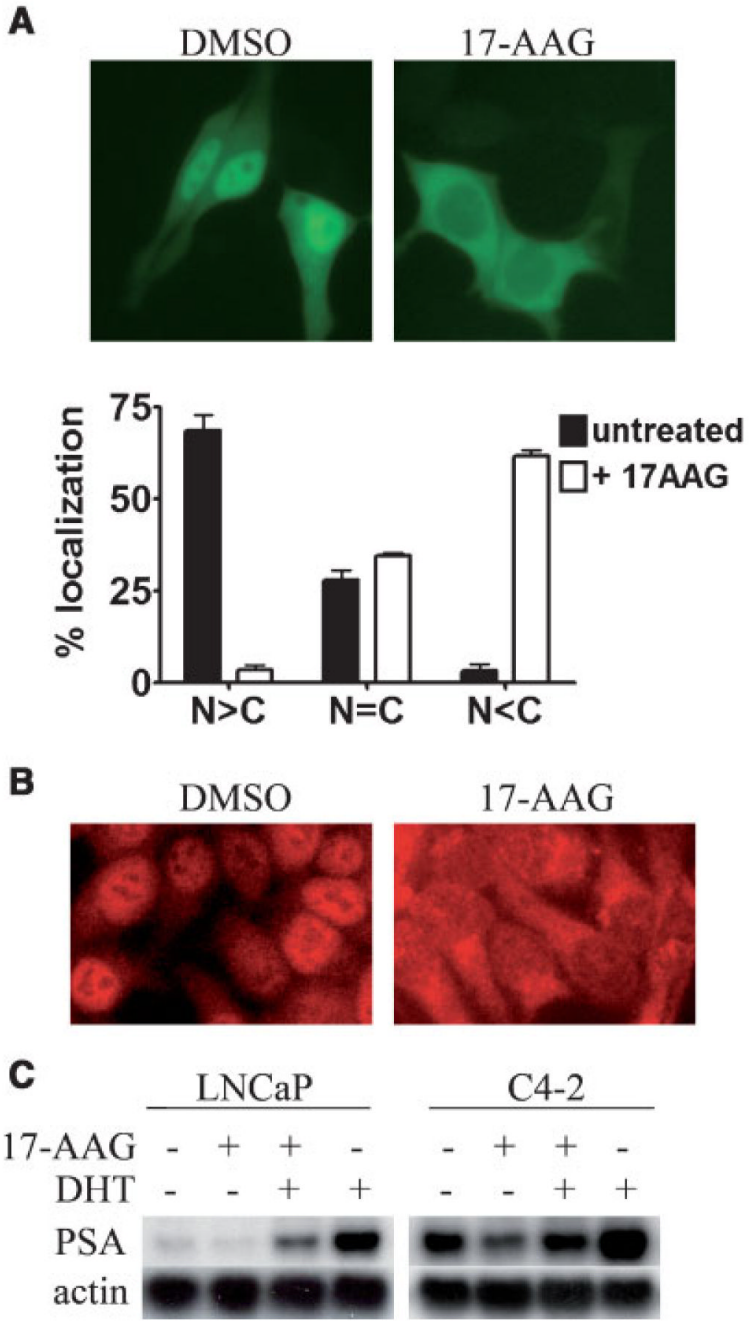

Fig. 4.

Effect of the hsp90 inhibitor 17-AAG on AR localization and activity in androgen-refractory prostate cancer cells. **A:** C4-2 cells were transfected with GFP-AR and treated with DMSO or 300 nM 17-AAG in ligand-free conditions. Localization was assessed by fluorescence microscopy 4 hr after treatment. Results are the average of four experiments, where >50 cells were analyzed for each experiment. **B:** Localization of endogenous AR in C4-2 cells treated with DMSO or 300 nM 17-AAG was determined by indirect immunofluorescence. **C:** LNCaP and C4-2 cells were pre-treated with 200 nM 17-AAG for 4 hr then 1 nM DHT was added to the medium containing 17-AAG, as indicated, and cells were cultured for an additional 24 hr.

RNA was isolated and PSA expression was determined by Northern blot analysis. β-actin mRNA is shown as a loading control.

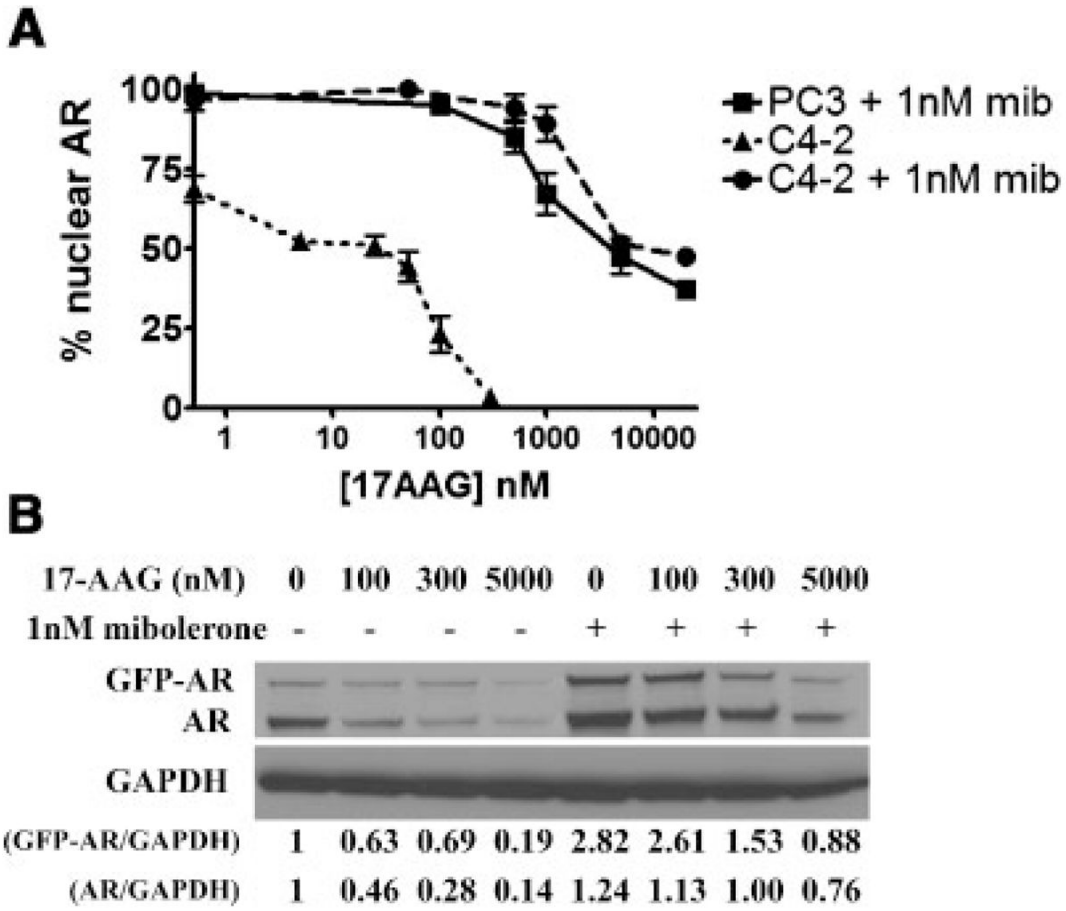

Fig. 5.

Ligand-independent nuclear localization and mibolerone-induced nuclear import exhibit different sensitivities to 17-AAG. C4-2 and PC3 cells were transfected with GFP-AR and treated with or without 1 nM mibolerone and the indicated doses of 17-AAG for 4hr. **A:** Localization of GFP-AR was determined by fluorescence microscopy. **B:** Effect of 17-AAG on GFP-AR and endogenous AR protein levels in C4-2 cells. C4-2 cell lysates were subjected to SDS–PAGE and GFP-AR and endogenous AR levels were determined by Western blot analysis. Densitometry was used to quantify the effects of 17-AAG and mibolerone on the relative ratios of GFP-AR and AR to the loading control, GAPDH.