Hsp90 inhibitor 17-AAG inhibits progression of LuCaP35 xenograft prostate tumors to castration resistance

Katherine J. O'Malley[1], **Gabrielle Langmann**[1], **Junkui Ai**[1], **Raquel Ramos-Garcia**[1], **Robert L. Vessella**[4], and **Zhou Wang**[1,2,3]

[1]Department of Urology, University of Pittsburgh, Pittsburgh, PA

[2]Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA

[3]University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA

[4]Department of Urology, University of Washington School of Medicine, Seattle, WA

Abstract

BACKGROUND—Advanced prostate cancer is currently treated with androgen deprivation therapy (ADT). ADT initially results in tumor regression, however, all patients eventually relapse with castration-resistant prostate cancer. New approaches to delay the progression of prostate cancer to castration resistance are in desperate need. This study addresses whether targeting HSP90 regulation of androgen receptor (AR) can inhibit prostate cancer progression to castration resistance.

METHODS—The HSP90 inhibitor 17-AAG was injected intraperitoneally into nude mice bearing LuCaP35 xenograft tumors to determine the effect of HSP90 inhibition on prostate cancer progression to castration resistance and host survival.

RESULTS—Administration of 17-AAG maintained androgen-sensitivity, delayed the progression of LuCaP35 xenograft tumors to castration resistance and prolonged the survival of host. In addition, 17-AAG prevented nuclear localization of endogenous AR in LuCaP35 xenograft tumors in castrated nude mice.

CONCLUSIONS—Targeting Hsp90 or the mechanism by which HSP90 regulates androgen-independent AR nuclear localization and activation may lead to new approaches to prevent and/or treat castration-resistant prostate cancer.

Keywords

Androgen Receptor; HSP90; Castration Resistance; LuCaP35

Introduction

Androgen deprivation therapy (ADT) is the standard treatment for patients with metastatic prostate cancer. Unfortunately, ADT is only palliative and patients will eventually relapse with castration-resistant prostate cancer. The current therapies for castration-resistant prostate cancer, including Docetaxol and Provenge, have limited efficacy and can only extend the life of patients an average of 2–4 months (1,2). New approaches to delay the progression of prostate cancer to castration resistance are desperately needed. Elucidating

the molecules involved in the progression of prostate cancer to castration resistance has significant clinical relevance.

Abnormal activation of androgen receptor (AR) is a key mechanism leading to castration-resistance in prostate cancer (3–5). The androgen receptor (AR), a member of the steroid receptor superfamily, is an androgen-dependent transcription factor that controls the expression of androgen-responsive genes (6). Intracellular trafficking represents a key step in the regulation of many transcription factors, including AR. In order to access its target genes, AR must be present in the nucleus. Thus, a key regulatory step in the action of AR is its nuclear localization (7–9). In androgen-sensitive cells, AR resides in the cytoplasm in the absence of ligand. Upon addition of androgens, AR translocates to the nucleus and transactivates target genes. However, in castration-resistant prostate cancer cells, AR remains in the nucleus even in the absence of androgen and transactivates androgen-responsive genes, leading to growth of prostate tumors under androgen-depleted conditions (10,11). Therefore, the development of novel approaches that can block the nuclear localization of AR may be an effective treatment strategy for prostate cancer. A recent study of Zhu et al showed inhibition of androgen-dependent AR nuclear translocation by tubulin-targeting chemotherapeutic agents (12), providing a new mechanism for docetaxel treatment of prostate cancer as well as substantiating the importance of targeting AR nuclear translocation in prostate cancer cells.

Heat shock protein 90 (HSP90) is an abundant chaperone protein involved in the regulation of many key signaling proteins and steroid receptors including AR (13–20). Recent studies showed that HSP90 plays an important role in the regulation of AR nuclear localization and activation in castration-resistant prostate cancer cells (20,21). Another study showed that inhibition of HSP90 by 17-AAG can induce degradation of AR and other important signaling molecules such as HER-2/NEU and inhibition of prostate xenograft tumor growth (16). 17-AAG can inhibit both AR-negative and –positive prostate cancer cells, suggesting that 17-AAG inhibition of tumor growth could bypass AR. Also, inhibition of AR in castration-resistant prostate cancer cells by 17-AAG is not equal to restoration of androgen-responsiveness of the tumor cells. Thus, it will be important to test whether HSP90 inhibition could maintain or prolong the sensitivity of prostate tumors to androgens via preventing androgen-independent nuclear localization and activation of AR under castration conditions.

Here we used a specific HSP90 inhibitor, 17-AAG, to investigate whether HSP90 inhibition could delay the progression of prostate cancer to castration resistance in the LuCaP35 xenograft tumor model. The LuCaP35 xenograft was established from lymph node metastases andexpresses wild-type AR and is sensitive to androgen deprivation (22). LuCaP35 xenograft tumors relapse after initial regression in response to castration in nude mice, providing an excellent model to address the importance of HSP90 in activation of AR under castration conditions and progression of prostate cancer to castration resistance.

MATERIALS AND METHODS

Animal Studies

Four to six week old balb/c nu/nu athymic male mice or SCID mice were purchased from Harlan Labs (Indianapolis, IN). Experiments were carried out under approval through an Institutional Animal Care and Use Committee approved protocol at the University of Pittsburgh. Guidelines for the proper and humane use of animals in research were followed. The mice were maintained one week for recovery from the shipment prior to experimentation. Anesthetized intact mice were implanted subcutaneously with 20–30 mm^3 tumor bits from LuCaP35 tumor propagated *in vivo* as previously described (22). Tumors

were measured three times weekly with micro-calipers, and tumor volumes were determined using the formula: (length × width^2)/2 (23,24).

Mice were randomized into three groups when tumor volume reached 200mm^3: testes intact, castrated with EPL (Egg Phospholipid) vehicle injection, and castrated with 17-AAG injection. EPL and 17-AAG were a generous gift of the NCI (Rockville, MD). 17-AAG or EPL was injected intraperitoneally beginning 10 days post-randomization for 5 days a week for 3 weeks. Blood samples were collected from the saphenous vein the week of castration, one week post-castration, and 5 weeks post-castration to monitor serum PSA levels. Beginning one week post-castration, mice were treated with vehicle alone or 25mg/kg 17-AAG five days per week for three weeks. Tumor volumes were monitored continuously until 150 days post-castration. Mice were sacrificed as indicated or when any one tumor length reached 20mm.

Immunofluorescent Staining

Tumors were harvested and fixed overnight at 4°C in 4% paraformaldehyde. Tissues were washed in PBS and allowed to sit in 30% sucrose overnight at 4°C prior to embedding in TBS Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, N.C.). Embedded tissues were stored at −80°C until sectioning. Tissues were sectioned at 10μm thickness onto charged slides. The slides were allowed to sit at room temperature for 30 minutes and then incubated in ice cold acetone for 5 minutes. Slides were incubated again at room temperature for 10 minutes and stored until imunostaining at −80°C. Before staining, slides were acclimated to room temperature for 10 minutes. They were incubated in 0.5% Triton X-100 in PBS for 10 minutes. Antigen Unmasking Solution (Vector Laboratories, Inc. Burlingame, CA) was used to retrieve antigen by microwaving in solution. Sections were washed 2 times in PBS and endogenous enzyme activity was blocked using 0.3% normal goat serum in PBS for 4 hours. AR antibody N-20 (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted (1:500) in blocking solution at manufacturer's instructions, applied to sections and incubated overnight at room temperature. The following day sections were washed 2 times in PBS and secondary antibody AlexaFluor conjugated goat anti-rabbit 647(Invitrogen Molecular Probes, Eugene OR) was added at 1:200 in 0.3% normal horse serum. Sections were incubated for 1 hour at room temperature followed by 3 washes with PBS. Vectashield (Vector Laboratories, Inc. Burlingame, CA) containing DAPI nuclear stain was used when viewing tissue sections. Photos were taken at 10 and 40× magnification on a Nikon T2000 microscope with NIS Elements Imaging Software version Br (Nikon, Melville, NY).

Statistics and Data Analysis

Statistics were calculated using Graphpad Software (Graphpad Software, Inc., La Jolla, CA) for Students T test, Fisher's Exact Test and Kaplan-Meier Test where stated. In this study, we defined statistically significant as p-value less than or equal to 0.05.

RESULTS

LuCaP35 xenograft tumors are androgen-sensitive

We first evaluated the androgen-responsiveness of LuCaP35 tumors being used in this study. As expected, serum PSA levels were significantly higher in the testes-intact animals compared to the other castrated groups at 2–3 days post-castration or randomization with the castrated animals showing an approximate 3-fold decrease in serum PSA (Fig. 1). These observations show the androgen-responsiveness of LuCaP35, which is in agreement with the previously studies (22).

17-AAG inhibits LuCaP35 tumor growth

Mice treated with 17-AAG for 5 weeks following castration caused a decrease of less than 30% in mean tumor volume, whereas animals treated with vehicle alone showed a steady increase in volume at 63% increase by week 5 post-castration (Fig. 2a). At the end of the 5-week treatment, the tumor volume in vehicle-treated animals was approximately 3 fold greater than that of the 17-AAG treated mice (p < 0.05). Thus, 17-AAG inhibited the growth of LuCaP35 tumors in castrated mice.

To determine that differences in tumor volume were not affected by other health issues, we measured body weight throughout the study. Figure 2b shows no significant change in body weight through study end at 21 weeks post-castration.

17-AAG treatment prolonged survival of mice bearing LuCaP35 tumors

A Kaplan-Meier survival analysis was done on each group out to 21 weeks post-castration. Animals were sacrificed when tumor volume reached 2000mm^3 or when any on length on each tumor reached 20mm at which time the mice were euthanized. By week 10 post-castration, 100% (12/12) of the testis-intact animals died or sacrificed due to excessive tumor burden. Of the vehicle-treated castrated group, more than half (7/12) of the mice died or sacrificed within 13 weeks post-castration. As expected, only 2 out of 12 mice in the 17-AAG-treated castrated group reached a tumor burden of 2000 mm^3 by 21 weeks post-castration (2/12) (Fig. 3). Thus, 17-AAG treatment prolonged survival of castrated animals bearing LuCaP35 tumors.

17-AAG inhibited progression of LuCaP35 xenograft tumors to castration resistance

To determine if 17-AAG inhibition of LuCaP35 tumor growth in castrated mice is due to the retardation of tumor progression to castration-resistance, we determined the serum PSA levels in mice 1 week after the completion of 17-AAG treatment and in control mice treated with vehicle. By week 5 following castration 35% of animals (6/17) in the vehicle alone treatment groups had progressed to castration-resistance defined by serum PSA measurements greater than 6 ng/ml, however, none of the 17-AAG-treated animals exhibited increased serum PSA (0% or 0/17) (Fig. 4). A Fisher's Exact log-rank test for these data showed a significant difference between those animals treated with 17-AAG and vehicle alone in progression to a castration-resistant state (*p<0.01). There should be virtually no 17-AAG left in mice by week 5 following castration, because 17-AAG injection was completed by week 4 and the half-life of 17-AAG is only several hours (25). The above observations indicate that transient 17-AAG treatment maintained the sensitivity of AR in LuCaP35 tumors in castrated hosts, delaying their progression to castration-resistance.

17-AAG prevented nuclear localization of endogenous AR in LuCaP35 xenograft tumors

Previous studies showed 17-AAG inhibition of AR nuclear localization in castration-resistant prostate cancer cells in vitro (21). However, it is not clear whether cytoplasmic localization of AR in androgen-sensitive prostate cancer cells in vivo in castrated mice can be maintained or prolonged after 17-AAG treatment. In order to determine the effects of 17-AAG on AR nuclear localization in LuCaP35 tumors, we performed AR immunostaining of LuCaP35 tumors from testis-intact mice or castrated mice treated with 17-AAG or vehicle. Not surprisingly, AR immunostaining in animals treated with 17-AAG or vehicle alone and previously defined as castration-sensitive and PSA negative was predominantly localized to the cytoplasm of the tumor cells (Fig. 5). The testes-intact animals or those treated with vehicle and previously defined as castration-resistant and PSA positive were found to have a trend toward AR in the nucleus or evenly distributed in the tumor cell (Fig. 5).

DISCUSSION

This study provided evidence that 17-AAG can preserve the sensitivity of AR signaling to androgens in LuCaP35 prostate tumors after castration and, as expected, can prolong the survival of animal hosts. Previous studies showed that 17-AAG can inhibit the growth of AR-negative androgen-independent, AR-positive androgen-sensitive, and AR-positive castration-resistant prostate tumors. However, the importance of AR in the inhibition of the AR-positive prostate tumor growth by 17-AAG is not clear and it has not been tested if 17-AAG can delay the progression of androgen-sensitive prostate tumors to castration-resistance. The results presented herein show that 17-AAG can delay the progression of androgen-sensitive LuCaP35 xenograft prostate tumor to castration-resistance and this delay of the progression is accompanied by the lack of androgen-independent AR nuclear localization, a key feature of castration-resistance.

LuCaP35 xenograft tumors provide an excellent model for prostate cancer progression to study the effect of 17-AAG on castration-resistance. LuCaP35 tumors contain wild-type AR and are sensitive to androgen manipulation (22). This is consistent with serum PSA reduction in newly castrated nude mice bearing LuCaP35 tumors (Fig. 1). However, some of the tumors relapsed quickly after castration and about 35% of the tumors became castration-resistant within 5 weeks after castration (Fig. 4). The heterogeneity of LuCaP35 xenograft tumors in their response to castration is consistent with the fact that prostate cancer is highly heterogeneous.

The mechanisms of Hsp90 action in prostate cancer are likely complex since Hsp90 has many client proteins such as Her2/neu, RB, various kinases and AR (16–19,21). 17-AAG can inhibit both AR-negative and –positive cells (16), raising a question on the role of AR in 17-AAG inhibition of AR-positive prostate cancer cells. The results in this study indicate that AR is an important mediator of 17-AAG inhibition prostate cancer progression to castration-resistance, because the sensitivity of AR to androgens was maintained in the presence of 17-AAG after castration (Fig. 4). This finding is consistent with previous finding of AR being a key determinant in castration-resistance (3–5). Hsp90 modulation on AR activity may involve direct and/or indirect interactions between Hsp90 and AR. Elucidating the mechanisms by which Hsp90 modulate castration-resistant AR nuclear localization may lead to additional targets for the treatment and/or prevention of castration-resistant prostate cancer.

A phase II clinical trial of 17-AAG in patients with castration resistant prostate cancer did not show any activity based on PSA response, probably in part due to toxicity that would limit the drug dosage (26). It is possible that new Hsp90 inhibitors with less toxicity and more favorable pharmacokinetics may be developed in the future and they could be used to prevent and/or treat castration-resistant prostate cancer. Previous studies showed that HDAC6 can regulate Hsp90 acetylation (27) and that Hsp90 deacetylation by HDAC6 is required for androgen-independent nuclear localization and activation of AR (28), suggesting that HDAC6 inhibition could also delay the progression of prostate cancer to castration-resistance. De Leon and colleagues recently reported targeting Hsp90 cochaperone FKBP52 in the regulation of AR signaling in prostate cancer cells, which may lead to another approach to inhibit castration-resistance (29).

In summary, the present study argues that Hsp90 is required in the progression of prostate cancer to castration-resistance. Future research aimed at elucidating the mechanism of HSP90 regulation of androgen-independent nuclear localization and activation of AR is warranted. Novel small molecules targeting Hsp90, factors such as HDAC6 that can regulate Hsp90, and/or the mechanisms by which HSP90 regulates androgen-independent nuclear

localization and activation of AR may lead to new therapies to treat and/or prevent castration-resistant prostate cancer.

Acknowledgments

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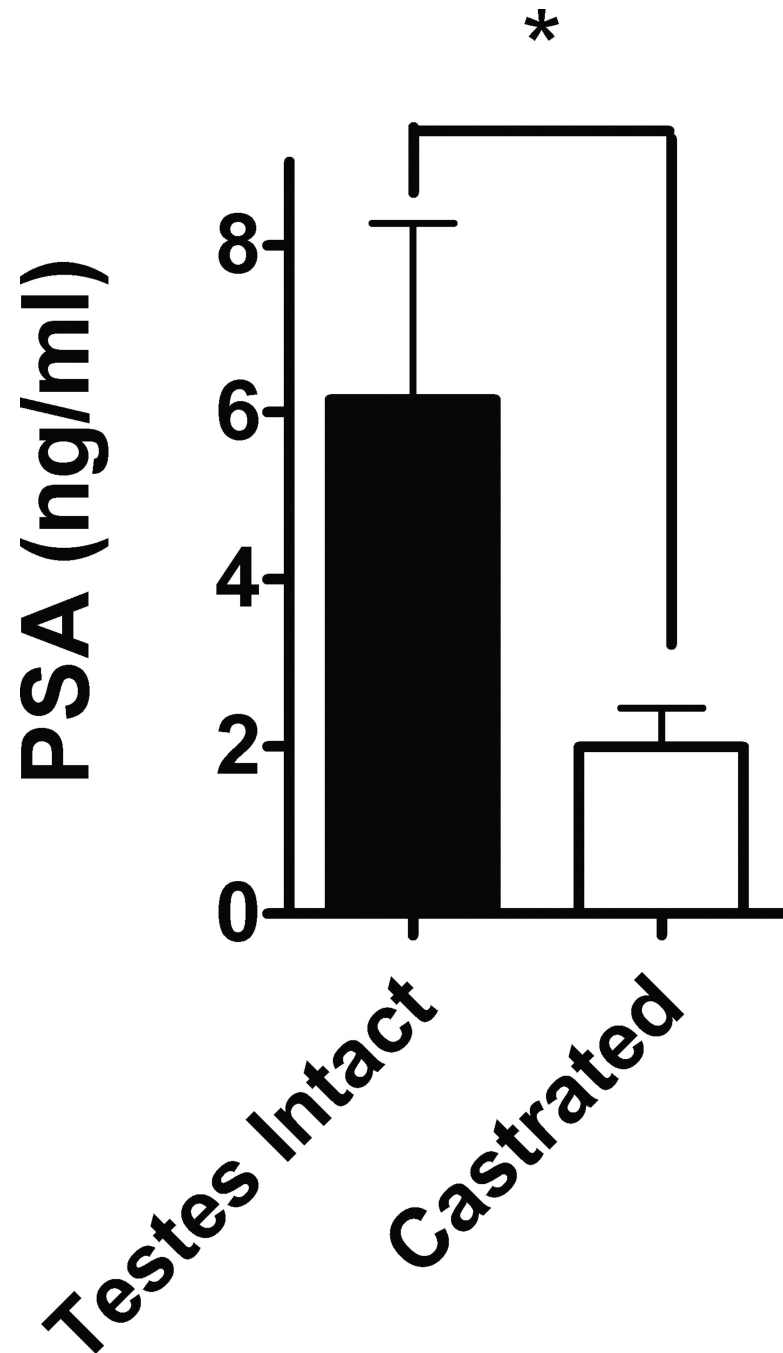
Fig. 1. Effects of castration on serum PSA of nude mice bearing LuCaP35 xenograft tumors
LuCaP35 tumor establishment and animal castration were described in Methods section.
Mean serum PSA (±SD) were measured 2–3 days post-castration. Numbers of animals for
testes intact and castrated were 5 and 32 respectively. *p<0.01
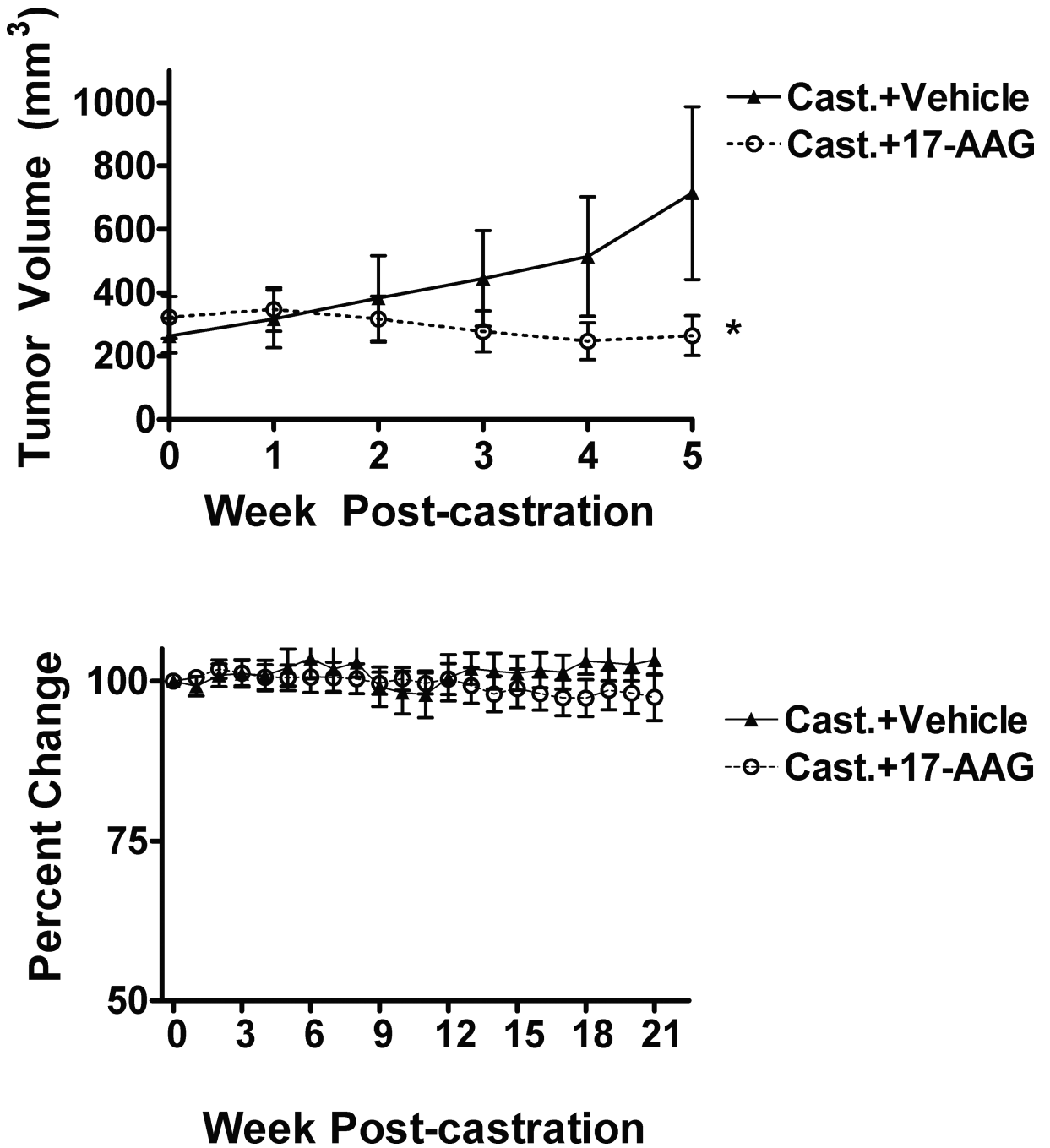
Fig. 2. Effects of 17-AAG on LuCaP35 xenograft tumor growth in castrated mice
(a) Mean tumor volume in castrated mice treated with vehicle (Cast.+Vehicle) or 17-AAG (Cast.+17-AAG) were measured at indicated time. The tumor volume difference between two groups was statistically significant (*p<0.05). Each group contains 17 animals. (b) Animal body weight was determined at indicated time and was normalized to the initial body weight. The number of animals was 12 for each group. Error bars represent standard deviation.
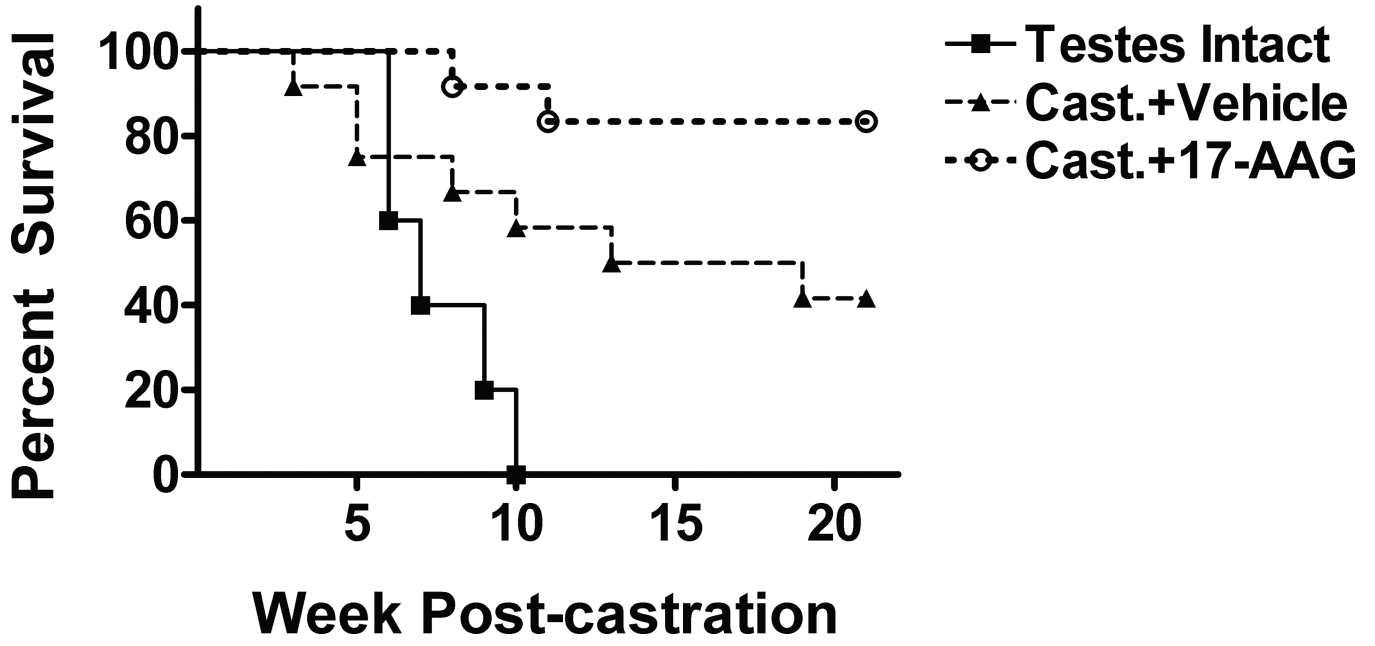
Fig. 3. Kaplan-Meier analysis of the effect of 17-AAG on survival of castrated mice bearing LuCaP35 xenograft tumors
Animals were randomized and castrated when LuCaP35 tumors reach 200 mm^3. Numbers of animals in Testis-Intact, Cast.+Vehicle, and Cast.+17-AAG were 5, 12 and 12 respectively. Euthanasia was performed if tumor diameter measured 20mm in any direction. The differences in survival were statistically significant according to the Log-rank test (*p<0.05).
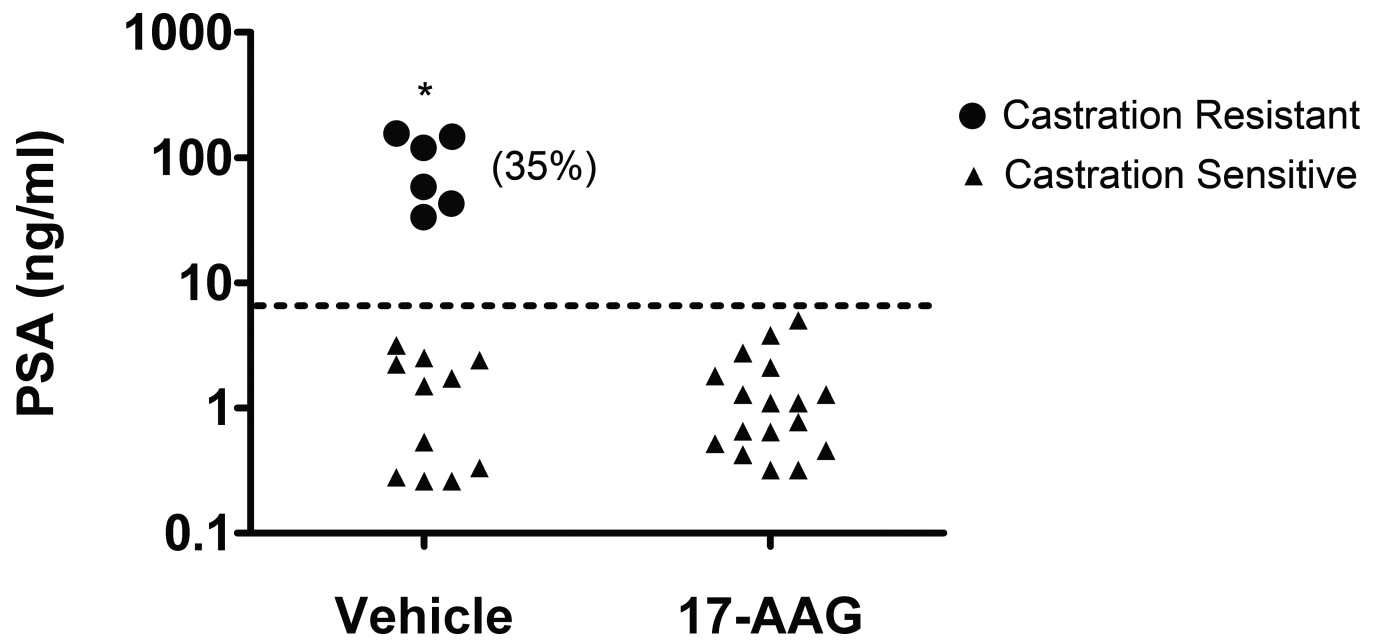
Fig. 4. 17-AAG prevention of LuCaP35 tumor progression to castration resistance
Serum PSA was measure to determine relapse of LuCaP35 tumors 5 weeks after castration, which was 1 week after the completion of 17-AAG or vehicle administration. A PSA value of 6 ng/ml, marked by the dotted line, is considered indicative of tumor relapse. Progression to castration resistance in the vehicle-treated group is statistically different from that in the 17-AAG-treated group according to Fisher's Exact Test (*p < 0.02). Each group contained 17 animals.
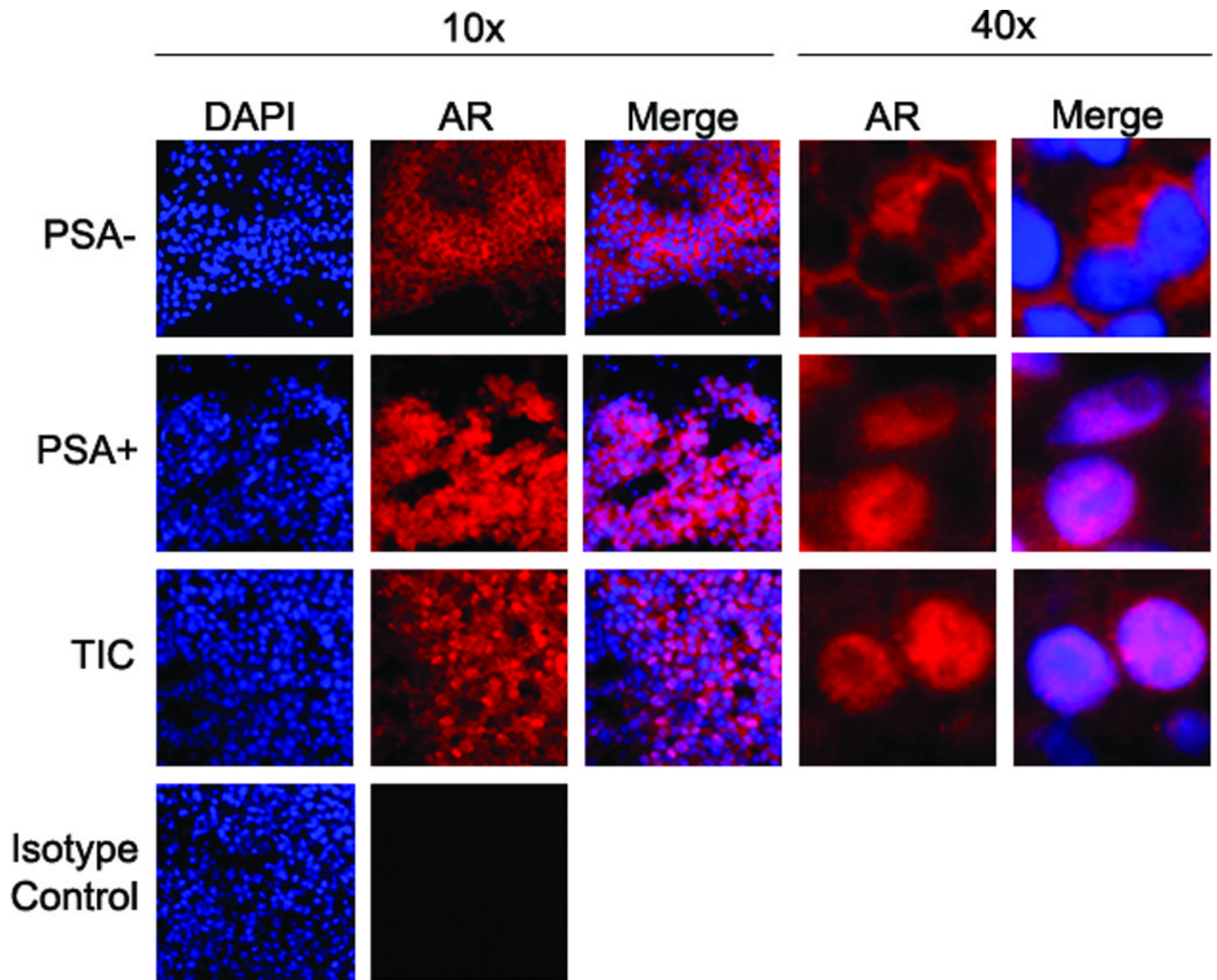
Fig. 5. Endogenous AR localization in LuCaP35 xenograft tumors
Endogenous AR subcellular localization in indicated LuCaP35 xenograft tumors was
determined by immunofluorescent staining as described in Methods. Nuclei were stained by
Hoechst. Images are the Tumors with serum PSA below 6ng/ml were considered PSA-
negative (PSA-), whereas those with serum PSA above 6ng/ml were PSA-positive (PSA+).
Tumors from testis-intact control mice (TIC) were included. Isotype control was stained
using rabbit IgG in place of primary AR antibody. Magnification of the objectives (10× or
40×) was indicated in the figure.