Berberine Suppresses Androgen Receptor Signaling in Prostate Cancer

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Abstract

The androgen receptor (AR) is critical in the normal development and function of the prostate, as well as in prostate carcinogenesis. Androgen deprivation therapy is the mainstay in the treatment of advanced prostate cancer; however, after an initial response, the disease inevitably progresses to castration-resistant prostate cancer (CRPC). Recent evidence suggests that continued AR activation, sometimes in a ligand-independent manner, is commonly associated with the development of CRPC. Thus, novel agents targeting the AR are urgently needed as a strategic step in developing new therapies for this disease state. In this study, we investigated the effect of berberine on AR signaling in prostate cancer. We report that berberine decreased the transcriptional activity of AR. Berberine did not affect AR mRNA expression, but induced AR protein degradation. Several ligand-binding, domain-truncated AR splice variants have been identified, and these variants are believed to promote the development of CRPC in patients. Interestingly, we found that these variants were more susceptible to berberine-induced degradation than the full-length AR. Furthermore, although the growth of LNCaP xenografts in nude mice was inhibited by berberine, and AR expression was reduced in the tumors, the morphology and AR expression in normal prostates were not affected. This study is the first to show that berberine suppresses AR signaling and suggests that berberine, or its derivatives, presents a promising agent for the prevention and/or treatment of prostate cancer.

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Introduction

Prostate cancer is the second most common cancer and a leading cause of cancer mortality among men in the United States (1). Androgen deprivation therapy (ADT), which aims to reduce the level of circulating androgens or to block the binding of androgens to their receptor, is a mainstay treatment for advanced prostate cancer. However, after a moderate, short-term response, the disease eventually progresses to castration-resistant prostate cancer (CRPC), which is a lethal and incurable. Therefore, preventing the transition to CRPC and treating CRPC effectively have become critical challenges for prostate cancer management.

The androgen receptor (AR), a member of the steroid nuclear receptor family, is activated upon binding to androgens. The AR signaling axis not only plays a crucial role in the normal growth of the prostate, but also promotes prostate cancer initiation and progression. Recent studies have shown that AR reactivation is the driving force for the development of CRPC. Several mechanisms have been proposed to explain the sustained AR activation in a low-androgen environment. First, AR amplification and AR overexpression, which were seen in 20% tumors from CRPC patients by gene expression analysis and immunohistochemistry assay (2, 3), could sensitize the receptor to low concentrations of androgens (4). Laboratory studies indicate that AR overexpression is necessary and sufficient to induce CRPC in a xenograft model (5). Second, AR mutations could alter its ligand-binding specificity and allow it to be activated by non-androgen steroids or increase its binding affinity for androgens (5–8). Third, tyrosine kinases could activate AR directly by phosphorylation (9, 10). Fourth, recent studies indicate that CRPC cells acquire the ability of intracellular synthesis of testosterone and dihydrotestosterone (DHT) from weak adrenal androgens (11) or from cholesterol (12–15). Recently, several AR alternative splicing variants that lack the ligand-binding domain (LBD)
but retain the DNA-binding domain have been identified in both xenografts and human tissues (16–19). These variants, referred to as AR-LBDs hereafter, have been shown to be transcriptionally active in the absence of androgens and drive androgen-independent growth of prostate cancer cells (17–19). Collectively, these studies provide new insights into the development of CRPC and underscore the importance of AR as a direct therapeutic target in all stages of prostate cancer.

Berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine chloride; BBR) is an isoquinoline alkaloid (Fig. 1A) with a long history of use in traditional medicine. It can be isolated from several plants of the genera *Berberis* and *Coptis*, including goldenseal, Oregon grape, and barberry. Recent studies have reported that BBR has anticancer activities against several types of tumors (20, 21), including prostate cancer (22, 23). In this study, we tested the hypothesis that BBR suppresses the AR signaling pathway, which has not been reported previously.

**Materials and Methods**

**Reagents**

The reagents MTT, sulforhodamine B, cycloheximide, and 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) were purchased from Sigma-Aldrich. R1881 and dihydrotestosterone (DHT) were obtained from Steraloids, Lipofectamine and Plus from Invitrogen, MG132 from the American Peptide Company, and the pan-caspase inhibitor z-VAD-fmk from Becton Dickinson Pharmingen. Berberine (purity >99%) was acquired from the Northeast Pharmaceutical Group and from Sigma-Aldrich.

**Cell culture and MTT assay**

Cells from LNCaP, 22Rv1, and PC-3 cell lines were obtained from the American Type Culture Collection at Passage 4. The LAPC-4 cell line was provided by Dr. C.L. Sawyers (24). C4–2B cells were provided by Dr. S. Koochekpour at Louisiana State University Health Sciences Center, New Orleans, LA. All cells used in this study were within 20 passages after receipt or resuscitation (<3 months of noncontinuous culturing). The requirement of androgen for growth was tested intermittently, and the expression of AR was assessed by Western blot analysis during the study. LNCaP, LAPC-4, 22Rv1, C4–2B, and PC-3 prostate cancer cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mmol/L glutamine, 100 Units/mL penicillin, and 100 μg/mL streptomycin. In experiments requiring androgen stimulation, LNCaP and 22Rv1 cells were cultured in phenol red–free RPMI 1640 medium supplemented with 10% charcoal-stripped FBS (cs-FBS). MTT assay was conducted in 96-well plates in octuplicate. Cells were seeded at a density of 3 × 10³ cells/well overnight, and treated with BBR for 24, 48, or 72 hours. IC₅₀ values of BBR were calculated using the software Origin 6.0.

**Apoptosis assay**

LNCaP cells were plated onto 96-well plates at a density of 6 × 10⁴ cells/well in RPMI 1640 medium supplemented with 10% FBS overnight, and treated with BBR for 24 hours. Apoptosis was detected by the Cell Death Detection ELISA Kit (Roche). Cell number was determined in parallel by sulforhodamine B assay and was used to normalize the apoptosis result. Data were expressed as fold of apoptosis induction over that of control.

**Transient transfection and reporter-gene assay**

LNCaP and 22Rv1 cells were seeded on 10-cm dishes at a density sufficient to become 80% to 90% confluent after

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**Figure 1.** Berberine inhibits prostate cancer cell growth and induces apoptosis. A. structure of berberine. B, BBR inhibited the growth of LNCaP. Cells were treated with various concentrations of BBR for 24, 48, or 72 hours. MTT assay was conducted to determine cell viability. The viability of control cells was set at 100%. The data plotted are mean ± SD; n = 8. C, BBR induced apoptosis in LNCaP. Apoptosis was quantitated by cell death ELISA in LNCaP cells treated with indicated concentrations of BBR for 24 hours. Data plotted are mean ± SD; n = 3. *, P < 0.05; **, P < 0.01.
24 hours. Transient transfection was carried out by using the Lipofectamine and Plus reagents according to the manufacturer’s instructions. Cells were transfected with 4 μg of the AR33 plasmid (single luciferase assay) or cotransfected with 4 μg AR33 plasmid and 200 ng pRL-TK plasmid (dual luciferase assay). After incubating with the transfection mixture for 4 hours, cells were trypsinized and re-plated in triplicate onto 24-well plates at a density of $6 \times 10^4$ cells/well in RPMI 1640 medium containing 10% cs-FBS. Cells were allowed to recover overnight before treatment with 0, 25, 50, or 100 μmol/L BBR in the presence or absence of 1 nmol/L DHT. For single-luciferase assay, LNCaP cells were lysed with 1X Reporter Lysis Buffer (Promega) at 6 or 24 hours after treatment, and luciferase activity was assayed by using the Luciferase Assay System (Promega). Protein concentration was determined by using the BCA Protein Assay Kit (Pierce). The luciferase activity was normalized by the protein concentration of the same sample. Dual luciferase assay was conducted when there was a need to compare results from different cell lines. The assay was conducted 24 hours after treatment using the Dual Luciferase Reporter Assay System (Promega). Reovila luciferase activity was used to normalize the activity of firefly luciferase.

**Quantitative reverse-transcription PCR**

Quantitative reverse-transcription PCR (qRT-PCR) was carried out as described previously (25). cDNA was synthesized from 1.5 μg of total RNA by the SuperScript III Reverse Transcriptase (Invitrogen). The mRNAs for AR, prostate-specific antigen (PSA), and 24-dehydrocholesterol reductase (DHCR24) were analyzed by the relative gene expression protocol, and β-actin was used as the internal control.

**Western blotting and immunoprecipitation**

Cells were washed with ice-cold PBS and lysed with 2× Cell Lysis Buffer (Cell Signaling) containing a phosphatase inhibitor and the protease inhibitor cocktail (Sigma) by incubating on ice for 30 minutes. Lysates were collected by centrifugation and protein concentrations were determined by the BCA method. For immunoprecipitation, cells were lysed with the lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 125 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP-40), and 1.2 mg of total protein were incubated with the anti-HSP90α/β antibody or the mouse immunoglobulin G (IgG; negative control) overnight at 4°C. Protein-G Agarose (Invitrogen) was added and the mixture was incubated for 1 hour at 4°C. The immunocomplex was precipitated by a magnetic rack, washed 4 times with the lysis buffer, and resuspended in the SDS-loading buffer. The samples were separated on 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After blocking in TBS buffer (150 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) containing 5% nonfat milk, the blots were incubated with a primary antibody overnight at 4°C and a fluorescent-labeled secondary antibody for 1 hour at room temperature. The fluorescent signals were obtained by the Odyssey Infrared Imaging System (LI-COR Bioscience). The primary antibodies used were as follows: anti-AR (Millipore), anti-PSA, anti-HSP90α/β (Santa Cruz Biotechnology), anti-DHCR24 (Cell Signaling), and anti-GAPDH (Chemicon).

**Immunocytofluorescence imaging**

LNCaP cells were cultured on poly-L-lysine-coated cover slides in phenol red–free RPMI 1640 medium plus 10% cs-FBS overnight and treated with BBR for 2 hour. R1881, a synthetic androgen, was added to make up a final concentration of 1 nmol/L. After 2.5 hours, cells were fixed, permeabilized, and incubated with an anti-AR antibody and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Images were captured at ×63 magnification using a Leica TCS SP2 confocal microscope.

**In vivo tumor growth in a xenograft model**

LNCaP cells ($4 \times 10^6$) were collected in 70 μL PBS and mixed with 70 μL Matrigel Matrix (Becton Dickinson Biosciences). The mixture was injected subcutaneously to one side of the dorsal flank of 6- to 7-week-old male nu/nu mice (National Cancer Institute, Frederick, MD). When tumor volume reached 100 mm³, mice were randomized into 2 groups ($n = 6$) and treated with vehicle (DMSO, 1 mL/kg) or BBR (5 mg/kg, dissolved in DMSO), respectively, by daily intraperitoneal (i.p.) injection. Tumor volumes were measured every 3 days and calculated using the formula: $r_1 \times r_2 \times 0.52$, where $r_1 > r_2$. Mice were sacrificed 15 days after treatment.

**Immunohistochemistry staining**

Tumor tissues were fixed in 10% buffered formalin and embedded in paraffin. The sections were dewaxed and rehydrated in graded ethanol. Antigen retrieval was carried out by soaking the sections in 10 mmol/L sodium citrate buffer (pH 6.0) and heating in a high-power microwave oven for 20 minutes. The sections were incubated with a mouse anti-AR monoclonal antibody (Biogenex) for 30 minutes at room temperature. After washing, the secondary antibody was added and incubated for 30 minutes. Reactive products were visualized by staining with 3,3′-diaminobenzidine and counterstained with hematoxylin for 30 seconds. Images covering the entire slide were captured with the same optical parameters. All images, excluding areas near the edges or with necrosis, were analyzed using the ImageJ Software (NIH). Cells with strong, medium, weak, or negative staining for AR were counted and the percentage of cells in each category was calculated.

**Statistical analysis**

All results are presented as mean ± SD unless otherwise stated. Statistical significance was determined with the Student’s t test (2-tailed). $p < 0.05$ was considered statistically significant.
Results

**BBR inhibits cell growth and induces apoptosis in prostate cancer**

We first used the MTT assay to investigate the effect of BBR on cell viability in several prostate cancer cell lines, including LNCaP, LAPC-4, 22Rv1, C4-2B, and PC-3. The AR-expression and androgen-dependence status of these lines are described in Table 1. Cells were treated with increasing concentrations of BBR (from 1.56 to 100 μmol/L in 2-fold increments) for 24, 48, or 72 hours. The MTT results show that BBR reduced the viability of all cell lines in a dose- and time-dependent manner (Fig. 1B for LNCaP, remaining data not shown). The IC_{50} values indicate that AR-positive cell lines are more sensitive to BBR than the AR-negative, androgen-independent PC-3 cells. These results indicate that BBR may exert its growth inhibitory effect by disrupting the AR signaling pathway.

Furthermore, we conducted apoptosis assay in LNCaP cells after 24 hours of BBR treatment. As shown in Fig. 1C, a dose-dependent induction of apoptosis was detected, starting at 12.5 μmol/L. This result confirms previous reports showing that BBR is a potent inducer of apoptosis in prostate cancer cells (23).

**BBR inhibits ligand-dependent and -independent AR transactivation**

On the basis of the MTT results, we set out to examine the influence of BBR on the transcriptional activity of AR. LNCaP cells were transiently transfected with an androgen response element (ARE)-luciferase reporter construct and cultured in the presence or absence of androgen. The basal activity of AR was very low in LNCaP cells, and cells were cultured in the presence or absence of the androgen response element (ARE)-luciferase reporter construct, cultured in an androgen-depleted condition, and treated with BBR for 24 hours. Consistent with being an androgen-independent cell line, the basal activity of AR was 10 times higher in 22Rv1 than that in LNCaP cells (Fig. 2B). BBR suppressed AR activity by greater than 90% in 22Rv1 cells, suggesting that BBR suppresses the constitutive AR activity.

In addition to the ARE-luciferase assay, we analyzed the influence of BBR on the expression of AR-regulated genes. PSA is a well-characterized target gene of AR. DHCR24 has been recently identified as an AR-regulated gene (25, 26). Figure 2C and D showed that, at the 24-hour time point, the mRNA and protein levels of both genes were reduced by BBR in LNCaP cells. This is consistent with the results from the ARE-luciferase assay. BBR also decreased PSA expression in C4-2B cells (Supplementary Fig. S1).

**BBR downregulates AR protein expression**

To investigate how BBR exerts its inhibitory effect on AR transactivation, we investigated whether BBR modulated the expression of AR. The qRT-PCR results showed that the AR mRNA level was not decreased by BBR after a 24-hour treatment (Fig. 3A). Instead, AR mRNA was increased by the 25 and 50 μmol/L concentrations. In contrast, AR protein was downregulated by BBR in a concentration- and time-dependent manner (Fig. 3B). A reduction of AR protein was first detected after 16 hours of treatment with 100 μmol/L BBR. After 24 hours, all 3 concentrations of BBR decreased expression of AR protein. These results indicate that BBR exerts an inhibitory effect on AR expression through mechanisms beyond the mRNA level.

In addition to LNCaP, we found that BBR decreased AR expression in other AR-positive lines, including 22Rv1 (Fig. 3C), LAPC-4, and C4-2B (Supplementary Fig. S2). This shows that BBR downregulation of AR protein is universal in prostate cancer cells. It is noteworthy that we detected a high abundance of AR isoforms in the range of 75 to 80 kDa in 22Rv1 cells. These AR isoforms have been shown to be alternative splicing products and lack the LBD (16–19). These variants have been found to be enriched in CRPCs (27) and strongly implicated in promoting the progression to CRPC (17, 19). Interestingly, we found that these AR isoforms were more

<table>
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<th>Cell lines</th>
<th>Androgen dependence</th>
<th>AR status</th>
<th>IC_{50} μM</th>
</tr>
</thead>
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<tr>
<td>LNCaP</td>
<td>Dependent</td>
<td>Mutant and functional AR</td>
<td>24.4</td>
</tr>
<tr>
<td>LAPC-4</td>
<td>Dependent</td>
<td>Wild-type AR</td>
<td>37.8</td>
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<td>22Rv1</td>
<td>Independent</td>
<td>Full-length (mutant) and truncated AR</td>
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<tr>
<td>C4-2B</td>
<td>Independent</td>
<td>Mutant and functional AR</td>
<td>49.4</td>
</tr>
<tr>
<td>PC-3</td>
<td>Independent</td>
<td>AR null</td>
<td>70.2</td>
</tr>
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Abbreviation: AR, androgen receptor.
susceptible to BBR than the full-length AR in 22Rv1 cells (Fig. 3C).

**BBR induces AR degradation**

The afore-described data led us to speculate that BBR may affect AR protein levels by reducing the half-life of AR. To test this possibility, LNCaP cells were pretreated with 50 μg/mL cycloheximide for 30 minutes to stop protein synthesis and then treated with 100 μmol/L BBR. Cells were lysed at different intervals for Western blotting analysis. Normalized AR protein levels were analyzed by linear regression to determine their half-life. As shown in Fig. 4A, AR half-life was 19.2 hours in control cells compared with 10.8 hours in cells treated with BBR, suggesting that BBR induces AR protein degradation.

It has been shown that AR could be degraded by 2 pathways, one is mediated by the proteasome (28) and the other by caspase-3 (29). To test the involvement of the proteasome pathway, LNCaP cells were treated with 100 μmol/L BBR for 8 hours before the proteasome inhibitor MG132 was added. Consistent with a previous report (30), MG132 increased AR level in the absence of BBR (Fig. 4B, lane 2). In cells treated with BBR, the presence of MG132 prevented further AR degradation (Fig. 4B, lanes 4 and 5).

To determine the involvement of a caspase-mediated pathway, we pretreated LNCaP cells with a pan-caspase inhibitor for 2 hours. Cells were then treated with BBR for 24 hours before being lysed for apoptosis assay and Western blotting analysis. As shown in Fig. 4C,
treatment with the inhibitor totally blocked apoptosis induction by BBR, suggesting that apoptosis induced by BBR is caspase-dependent. The AR protein was reduced by BBR to similar extents in the presence or absence of the inhibitor, indicating that BBR-induced AR degradation was not mediated by a caspase-dependent pathway. Furthermore, this suggests that AR degradation is a direct effect of BBR, rather than a bystander effect of apoptosis. Altogether, these results suggest that BBR-induced AR-protein degradation is mediated predominantly through the proteasome pathway.

**BBR disrupts AR-Hsp90 interaction**

Similar to other steroid nuclear receptors, unliganded AR resides in the cytoplasm and interacts with heat shock protein 90 (Hsp90), a major chaperone protein in the cytoplasm (31). This interaction stabilizes AR (32) and maintains the proper conformation of AR for high-affinity ligand binding (33). Upon ligand stimulation, AR undergoes conformational changes which lead to its dissociation with Hsp90, and subsequently translocates to the nucleus to function as a transcription factor. Disruption of the AR–Hsp90 interaction will not only render AR susceptible to protein degradation (32, 34) but also interfere with nuclear translocation (35, 36). We next examined the interaction between AR and Hsp90 by coimmunoprecipitation. LNCaP was cultured in a low-androgen condition for 48 hours, and then treated with 100 μmol/L BBR for 2 hours. Whole-cell lysates were immunoprecipitated by an anti-Hsp90 antibody. Figure 5A shows that the AR-Hsp90 association was significantly disrupted by BBR. Western blotting using input cell lysates showed that neither AR nor Hsp90 level was decreased by BBR under this condition.

**BBR inhibits AR nuclear translocation**

The effect of BBR on subcellularly distributed AR was examined by immunocytofluorescence staining. As shown in Fig. 5B, in the absence of androgen, AR staining was predominantly cytoplasmic. Following stimulation with R1881, the nuclear staining of AR was increased dramatically. AR staining in the nucleus was reduced in a dose-dependent manner in cells pretreated by BBR and accompanied by increased

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Figure 3. BBR downregulates AR protein expression. Cells were cultured in RPMI 1640 medium containing 10% FBS. A, qRT-PCR analysis of AR mRNA in LNCaP following BBR treatment for 24 hours. *, *P < 0.05; **, *P < 0.01. B and C, AR immunoblotting in LNCaP cells (B) and 22Rv1 cells (C) treated with 100 μmol/L BBR.
staining in the perinuclear cytoplasm. These results suggest that BBR inhibits AR nuclear translocation and provide an explanation to our observation that BBR inhibited AR activity before AR-protein level was reduced.

BBR inhibits tumor growth and suppresses AR in vivo
To test the in vivo efficacy of BBR, we first established LNCaP xenografts in nude mice. BBR treatment started when tumors reached 100 mm³ at a dose of 5 mg/kg/d. Figure 6A shows that treatment with BBR significantly inhibited the growth of the tumor xenografts, and this result was confirmed by the final tumor weights (Fig. 6B). No toxicity was observed in mice treated with BBR, as body weight changed similarly in both groups (Supplementary Fig. S3). The expression of AR in tumors was analyzed by immunohistochemical staining of formalin-fixed tissues. AR staining was found predominantly in the nucleus, both in the control and treatment samples (Fig. 6C). A decrease in AR-staining intensity was visually apparent in samples from the treatment group. Quantitative analysis showed that BBR reduced the percentages of cells with strong or medium staining of AR, and increased the percentages of cells stained weakly or negatively for AR. These results confirmed our cell-culture data and showed that BBR is effective in suppressing AR in vivo. Furthermore, the growth-inhibitory and AR-downregulating effects of BBR seems to be limited to the tumors, as BBR treatment does not affect the histology or AR expression in normal prostate glands of mice (Fig. 6D).
Discussion

The development of CRPC following androgen deprivation is the most critical challenge in the clinical management of prostate cancer. In spite of the recent development of new therapeutic options, treatment of patients with CRPC remains a significant clinical challenge. For example, sipuleucel-T (Provenge), the first therapeutic cancer vaccine approved by the Food and Drug Administration, has been shown by clinical trials to prolong the median survival of patients with metastatic CRPC by 4.1 months (37). Abiraterone, an inhibitor of the rate-limiting enzyme CYP17 in androgen biosynthesis, has shown promises in clinical trials involving patients with metastatic CRPC (38, 39). However, its efficacy against CRPC driven by the newly discovered AR splice variants has not been established. The lack of LBD could render these AR variants resistant to current androgen-blockade strategies. Therefore, suppressing the expression of all forms of AR appears to be a provocative and plausible strategy against CRPC.

Bioactive natural products provide a rich source for pharmacologic discovery. Berberine has been shown to induce apoptosis in prostate cancer cells by inducing reactive oxygen species (ROS) and oxidative stress (40). Our discovery that BBR induces AR degradation appears to be a novel mechanism that is independent of these actions of BBR. First, as shown in Fig. 4C, treatment with a pan-caspase inhibitor blocked apoptosis induction by BBR but had no impact on AR degradation, suggesting that AR degradation is not a result of destruction of cellular proteins during apoptosis. Second, AR degradation by BBR was not affected by treatment with a ROS scavenger, N-acetyl-L-cysteine (Supplementary Fig. S4). In LNCaP cells, it has been shown that BBR induced apoptosis by activating p53 (22). Therefore, at least in LNCaP cells, BBR appears to modulate 2 important signaling pathways in prostate cancer in a highly coordinated manner, that is, suppression of the prosurvival AR pathway and induction of the proapoptotic p53 pathway.

Our observation that BBR inhibited AR transactivation before the decrease in AR protein could be explained by the disruption of AR–Hsp90 interaction and reduction of AR nuclear translocation by BBR. These events were observed as early as 2 hours and 4 hours after treatment, respectively. The reduced AR–Hsp90 interaction is probably a result of decreased chaperone activity of Hsp90, as the level of Hsp90 protein was not affected by BBR at this time point. The activity of Hsp90 is regulated by the acetylation status, which, in turn, is regulated by histone deacetylase 6 (HDAC6). HDAC6 deacetylates the lysine residues of Hsp90 and plays a critical role in maintaining the chaperone function of Hsp90 (41). HDAC6 inhibition inactivates Hsp90 by interfering with ATP binding and association with client proteins (42). The molecular effects of BBR we have observed, including downregulation of the expression, transcriptional activity and nuclear localization of AR, and disruption of the AR/Hsp90 interaction, are similar to those achieved by HDAC6 inhibition (41, 43). Therefore, it is possible that BBR exerts its inhibitory action on AR through modulation of the HDAC6/Hsp90 pathway.

The client proteins of Hsp90 include steroid hormone receptors, including the estrogen receptor (ER), glucocorticoid receptor, and progesterone receptor. The possibility that BBR inhibits Hsp90 activity implies that BBR could have similar effects on these receptors. Indeed, we found that BBR reduced the protein level of ERs in MCF-7 breast cancer cells (Supplementary Fig. S5). Consistent with our finding, it has been shown that BBR enhances the anticancer efficacies of ER antagonists in ER-positive MCF-7 cells, but not in ER-negative MDA-MB-231 cells.
The effect of BBR on the ER signaling pathway warrants further investigation. Consistent with a previous report (22), our animal study showed that BBR is very effective in inhibiting the growth of prostate cancer in a xenograft model. The 5-mg/kg dose used in this experiment is well below the LD50 of BBR via the same route of injection, which is approximately 50 mg/kg (45). It is important to point out that, in spite of the fact that this dose was expected to achieve a blood concentration of BBR 2 orders of magnitude lower than the doses used in the cell culture experiments, our in vitro findings that BBR reduced AR protein and the expression of AR-regulated genes were confirmed by the animal experiment. Similar blood concentrations of BBR can be achieved in humans taking oral doses of BBR (46). Therefore, the findings described in this report are potentially clinically relevant.

In 22Rv1 cells, the basal activity of AR is 10 times that observed in LNCaP cells (Fig. 2B). Because 22Rv1 cells express high levels of ARALBDs (Fig. 3C), this ligand-independent AR transactivation is presumably driven by the splice variants. The data in Fig. 2B show that BBR is particularly effective in shutting down the ligand-independent AR activity in 22Rv1 cells, which is further supported by the data in Fig. 3C that BBR decreased the expression of ARALBDs more effectively than the full-length AR. In addition to BBR, several other natural or synthetic compounds have been shown to have similar inhibitory effects on AR slice variants (46–49). The discovery of these compounds should generate excitement because there is no drug currently in clinical trials to target CRPC progression driven by the AR splice variants. Our study suggests that BBR should be further tested and developed in the treatment of CRPC.
In summary, this work is the first report that BBR suppresses the AR signaling pathway in prostate cancer. BBR decreased AR transcriptional activity and reduced the expression of AR-regulated genes in both androgen-dependent and CRPC cells. AR protein was degraded by BBR, which was predominantly mediated by a proteasome pathway. Moreover, the disruption of AR–Hsp90 interaction and the inhibition of AR nuclear translocation by BBR could contribute to the depressed AR transcriptional activity and AR protein degradation. Interestingly, BBR induced degradation of not only the full-length AR, but also the ARALBDs. The animal data showed that BBR exerted growth-inhibitory and AR-downregulating effects specifically in tumor tissues. Our study identifies a novel mechanism for the anticancer effect of BBR and provides support for the use of BBR as a potential preventive and therapeutic agent in prostate cancer, particularly for AR-mediated CRPC growth.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

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