

# Silymarin inhibits function of the androgen receptor by reducing nuclear localization of the receptor in the human prostate cancer cell line LNCaP

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A number of reports have shown that the polyphenolic flavonoid silymarin (SM) is an effective anticancer agent. Agents with novel mechanisms of blocking androgen receptor (AR) function may be useful for prostate cancer prevention and therapy. Previous studies showed that silibinin (SB), the major active component of SM, could inhibit cell proliferation of a human prostate cancer cell line, LNCaP, by arresting the cell cycle at the G<sub>1</sub> phase without causing cell death. This study further delineates the potential molecular mechanism by which SM and SB exhibit antiproliferative effects on androgen-responsive prostate cancer cells by inhibiting function of the AR. We observed that SM and SB inhibited androgen-stimulated cell proliferation as well as androgen-stimulated secretion of both prostate-specific antigen (PSA) and human glandular kallikrein (hK2). Additionally, for the first time, we show that an immunophilin, FKBP51, is androgen regulated and that this up-regulation is suppressed by SM and SB. We further demonstrate that transactivation activity of the AR was diminished by SM and SB using gene transfer of PSA promoter and hK2 androgen-responsive element constructs. However, expression and steroid-binding ability of total AR were not affected by SM in western blotting and ligand-binding assays. Intriguingly, we found that nuclear AR levels are significantly reduced by SM and SB in the presence of androgens using western blotting assay and immunocytochemical staining. This study provides a new insight into how SM and SB negatively modulate androgen action in prostate cancer cells.

## Introduction

Prostate cancer is the most common type of cancer found in American men. The development and progression of prostate cancer may be related to overstimulation of the androgen receptor (AR) by androgens (1,2). Modulating AR function properly could be a very effective means for preventing the development and progression of prostate cancer.

Recently a number of reports have shown that the polyphenolic flavonoid silymarin (SM) is an effective anticancer agent (3–6). SM, a flavonoid extract from milk thistle (*Silybum marianum*) with extremely low toxicity (7), has been used clinically for alcoholic liver disease treatment in Europe and

Asia for almost 2000 years (8). A recent study showed that silibinin (SB), the major active component of SM, could arrest the cell cycle at the G<sub>1</sub> phase by alteration of several cell cycle-related proteins and without causing cell death in a human prostate cancer cell line, LNCaP (6). SB decreased androgen-stimulated growth and prostate-specific antigen (PSA) levels in LNCaP cells (6). In this study, we further evaluated the effects of SM and SB on androgen action. We found that SM and SB down-regulate several androgen-regulated genes, including PSA, human glandular kallikrein (hK2) and an immunophilin, FKBP51, mainly by inhibiting the transactivation activity of the AR. More interestingly, we show that SM and SB can inhibit nuclear localization of the AR. Thus this report provides a novel mechanism by which SM and SB inhibit function of the AR.

## Materials and methods

### Cell culture

Human prostate cancer cell line LNCaP (American Type Culture Collection) was grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) and 5% CO<sub>2</sub> at 37°C until ~50–70% confluence was reached. The medium were changed to serum-free RPMI 1640 to deplete undesired steroids for 24 h prior to experiments. Cells were then treated with RPMI 1640 supplemented with 5% charcoal-stripped FBS containing SM or SB (all from Sigma, dissolved in DMSO) at designated concentrations with or without 1 nM mibolerone (Mib) (from New England Nuclear, dissolved in ethanol), a non-metabolizable synthetic androgen. Equivalent amounts of solvent were added to control cells.

### Cell proliferation assay and PSA and hK2 quantification assays

LNCaP cells were seeded at 4×10<sup>4</sup>/well in 24-well dishes and treated with SM and SB at designated concentrations in the presence of 1 nM Mib. Five days later cell proliferation was measured with a MTS assay kit (Promega) and PSA and hK2 levels in spent medium were determined with a Tandem-E PSA kit (Hybritech) or by Mayo hK2 assay (9).

### Western blot analysis

LNCaP cells were treated with designated concentrations of SM or SB in the presence of 1 nM Mib for 24 h. Then cells were harvested and whole cell lysate and nuclear extract were prepared as described previously (10,11). Western blotting analysis was performed according to the protocol described previously (10). A mouse antibody against human FKBP51 (a gift from Dr David Toft, Mayo Clinic), human AR (Pharmingen) or human Sp1 (Santa Cruz) was used as the primary antibody. Ponceau S staining was used to monitor protein loading and transfer efficiency (12).

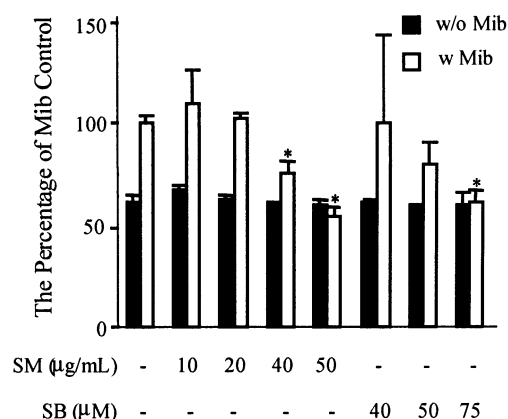
### DNA constructs

The 6 kb PSA promoter and the AR promoter constructs have been described previously (10). To make an hK2 androgen-responsive element (ARE) construct a DNA fragment containing three copies of the hK2 ARE (5'-ggaacatattgtatt-3') was synthesized by the Mayo Molecular Core Facility. The synthesized fragment, with restriction enzyme cutting sites for *SacI* and *XhoI* at the 5'- and 3'-ends, respectively, was digested accordingly and inserted into a pGL3-Promoter vector (Promega). The identity of this construct was confirmed by DNA sequencing.

### Transient transfection assay

LNCaP cells in duplicate plates were co-transfected with a CMV-β galactosidase (β-gal) expression vector (0.3 μg/plate) and a pGL3-Basic luciferase vector (Promega) containing the PSA promoter (–5824/+12), a pGL3-Promoter luciferase vector (Promega) containing three copies of the hK2 ARE or respective empty/parental vector using liposomes containing dimethyldioctadecyl ammonium bromide (Sigma) and L-lecithin (Sigma) (4:10). Cells were then treated with SM or SB in the absence or presence of 1 nM Mib

**Abbreviations:** AR, androgen receptor; ARE, androgen-responsive element; β-gal, β-galactosidase; FBS, fetal bovine serum; hK2, human glandular kallikrein; Mib, mibolerone; PBS, phosphate-buffered saline; PSA, prostate-specific antigen; SB, silibinin; SM, silymarin.



**Fig. 1.** Effects of SM and SB on androgen-regulated LNCaP cell growth. LNCaP cells were treated with SM and SB at designated concentrations in the absence or presence of 1 nM Mib for 5 days and cell proliferation was measured by MTS assay. Error bars indicate the standard errors of four separate experiments. \* $P < 0.05$ , when compared with the group treated with Mib alone.

for 24 h. Cell extracts were prepared and used for luciferase and  $\beta$ -gal assays (Promega). The  $\beta$ -gal activity was used for transfection control. The above experiments were repeated three times.

#### Ligand-binding analysis

LNCaP cells were plated in culture dishes at the same cell number and treated with SM or SB in the presence of 1 nM [ $^3$ H]Mib (New England Nuclear) with or without 100 nM cold Mib for 24 h. Then cell pellets were washed twice with 1× phosphate-buffered saline (PBS) and incubated with 95% ethanol for 30 min at room temperature. The ethanol-extracted supernatants were then removed for scintillation counting. The difference in radioactivities between groups with and without cold Mib represents the specific ligand-binding activity of the AR. The mean difference from triplicate measurements is presented.

#### Immunocytochemical staining

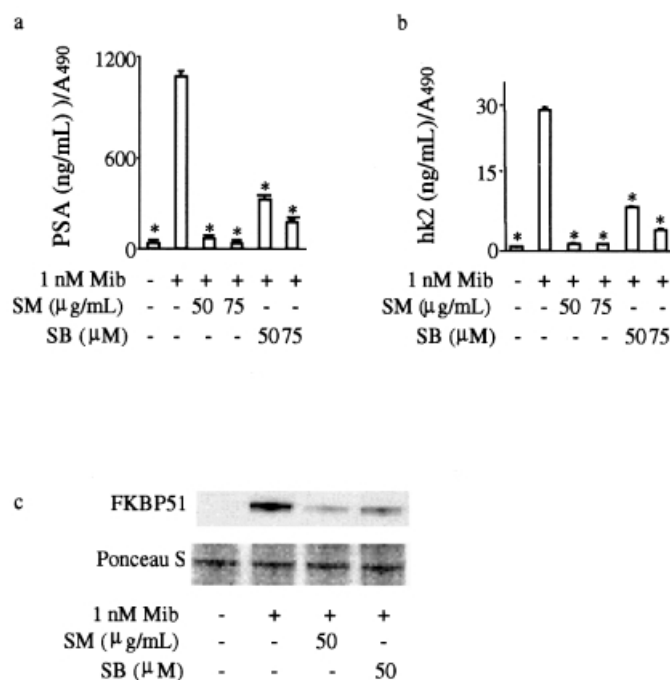
LNCaP cells grown in glass slide chambers (Sigma) were treated with SM and SB at the designated concentrations in the absence or presence of 1 nM Mib. Twenty-four hours later LNCaP cell slides were washed with 1× PBS and fixed in acetone at  $-20^{\circ}\text{C}$  for 10 min. The air-dried slides were treated with ethanol/chloroform (1:1) mixture for 5 min to permeate the cell membrane and air dried again. Then the slides were washed with 1× TBST and stained with a monoclonal antibody against human AR (Pharmingen) using a Dako kit. AR staining was observed under a microscope (Zeiss). The density of AR in a nucleus (or a whole cell) and the area of the nucleus (or the whole cell) were quantified by KS400 software (Zeiss), provided by the Mayo Molecular Core Facility. The AR protein level in a nucleus (or a whole cell) was calculated by multiplication of the density of AR in the nucleus (or the whole cell) and the area of the nucleus (or the whole cell). In each sample 20–25 cells were analyzed.

#### Statistics

Results were analyzed by two-tailed Student's  $t$ -test.  $P < 0.05$  was accepted as the level of significance.

## Results

To evaluate the effects of SM and SB on LNCaP cell growth, 1 nM Mib and various concentrations of SM or SB were added to the cultures. Cell growth was quantified by MTS assay 5 days later. Mib increased cell growth significantly (Figure 1). However, both SM and SB inhibited androgen-stimulated cell growth (Figure 1). Additionally, neither SM nor SB affected cell proliferation in the absence of Mib (Figure 1). SM at a concentration of 50  $\mu\text{g}/\text{mL}$  or SB at a concentration of 75  $\mu\text{M}$  fully suppressed androgen-stimulated cell growth (Figure 1). These results seem to be in agreement with the reported finding that SB inhibited LNCaP cell growth without causing cell death, although the effects of SM and SB on AR-mediated cell growth were not addressed (6).

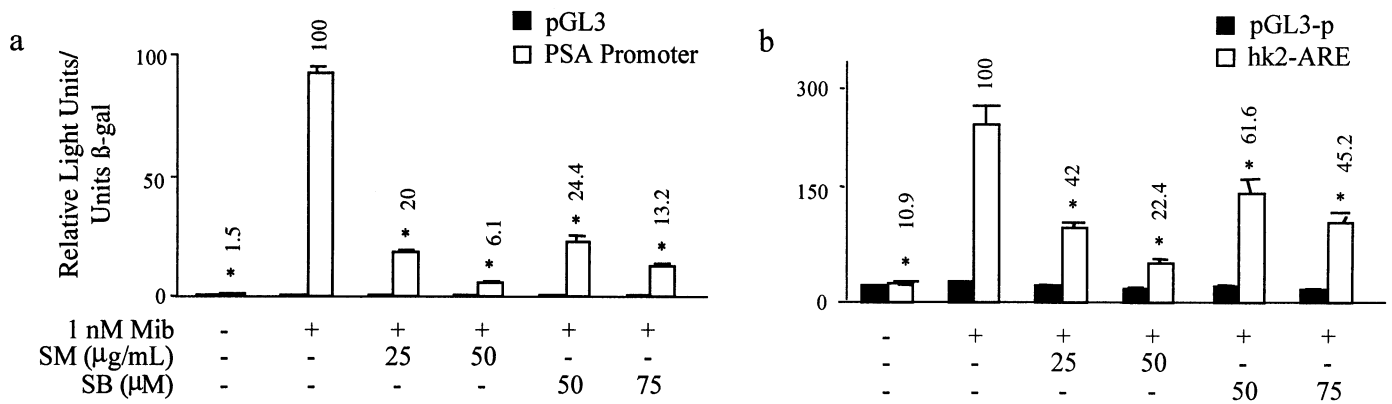


**Fig. 2.** Effects of SM and SB on expression of androgen-regulated proteins PSA, hK2 and fkbp51. (a) Secretion level of PSA; (b) secretion level of hK2; (c) western blot analysis of FKBP51 protein level. In the PSA and hK2 quantification assays LNCaP cells were treated with SM and SB at designated concentrations in the presence of 1 nM Mib for 5 days and PSA and hK2 secretion levels were normalized by variable cell number. Error bars indicate the standard errors of four separate experiments. \* $P < 0.05$ , when compared with the group treated with Mib alone. In western blot analysis LNCaP cells were treated with SM and SB in the presence of 1 nM Mib for 24 h.

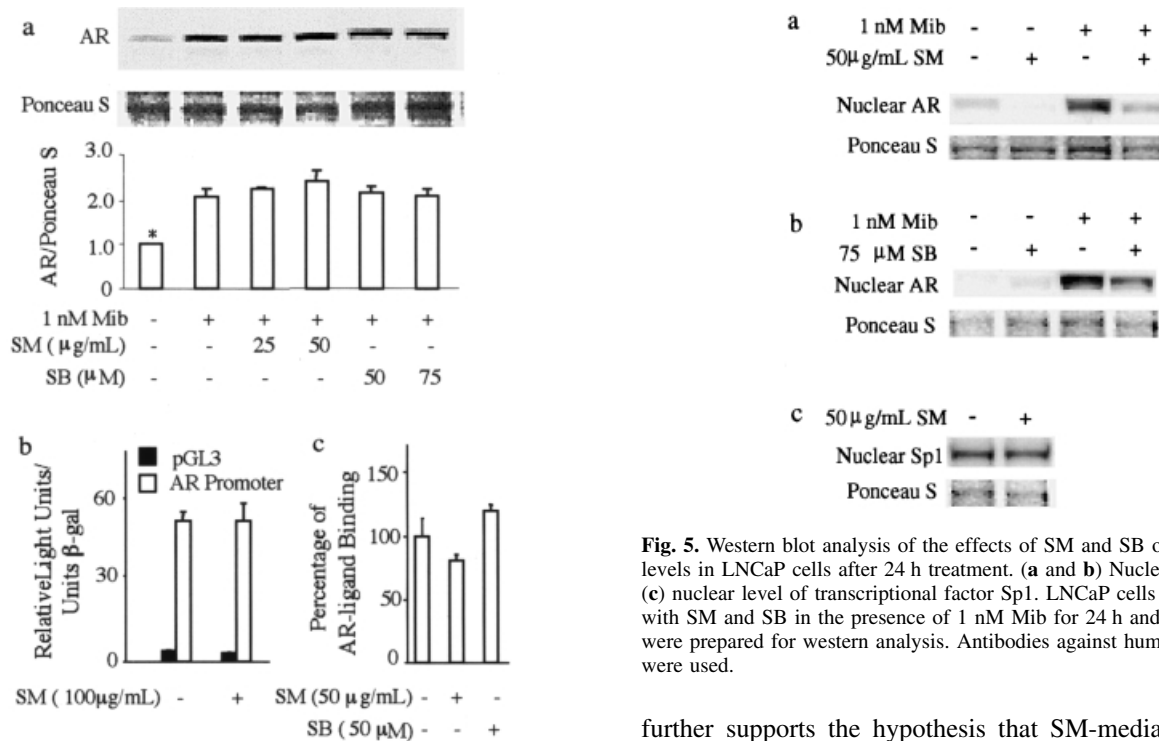
The prostate-specific kallikrein genes PSA and hK2 are primarily androgen regulated (13) and have been used as monitors for androgen action. Their promoters contain AREs for AR binding. As shown in Figure 2a and b, SM and SB inhibited PSA and hK2 secretion in LNCaP cells stimulated by androgens.

Recently we identified a novel androgen-regulated gene, FKBP51, using subtraction hybridization cloning technology (unpublished data). FKBP51, an FK506-binding immunophilin, can be detected in functionally mature steroid receptor complexes along with Hsp90 and p23 (14). Presently its function with the AR is not clear. Nevertheless, we found, for the first time, that FKBP51 protein level was up-regulated by androgens in LNCaP cells (Figure 2c). Both SM and SB treatment reduced androgen-enhanced FKBP51 protein level (Figure 2c).

Since the AR is the major regulator of PSA expression, we transfected a luciferase reporter gene containing the PSA promoter into LNCaP cells in order to determine the mechanism by which SM and SB affect androgen-regulated gene expression. SM and SB significantly reduced androgenic inducibility of the PSA promoter (Figure 3a), suggesting that AR function may be impaired by SM or SB. A luciferase reporter gene construct containing a minimum SV-40 promoter and three copies of the hK2 ARE was also transfected into LNCaP cells to investigate whether SM and SB inhibit function of the AR. As shown in Figure 3b, SM and SB inhibited ARE-regulated luciferase activity. These results strongly suggest that SM and SB inhibit AR-mediated transcription activity and seem to indicate that SM exerts about twice as strong an inhibitory effect as SB.



**Fig. 3.** Effects of SM and SB on 6 kb PSA promoter androgenic inducibility (a) and hK2 ARE-mediated transcriptional activity (b) in LNCaP cells. LNCaP cells were transiently co-transfected with a β-gal expression vector and a designated construct and then treated with SM and SB in the presence of 1 nM Mib for 24 h. Cell lysates were prepared for measurement of luciferase activities. Luciferase activities were measured and normalized to β-gal activities and expressed as relative light units/unit β-gal. Error bars indicate the SEM of three separate experiments. \* $P < 0.05$ , when compared with the group treated with Mib alone. A percentage of activities are also expressed at the top of some histograms as shown using the PSA promoter or hK2-ARE construct treated with Mib as a reference (expressed as 100). PGL3, a pGL3-Basic luciferase vector; PGL3-p, a pGL3-Promoter luciferase vector.



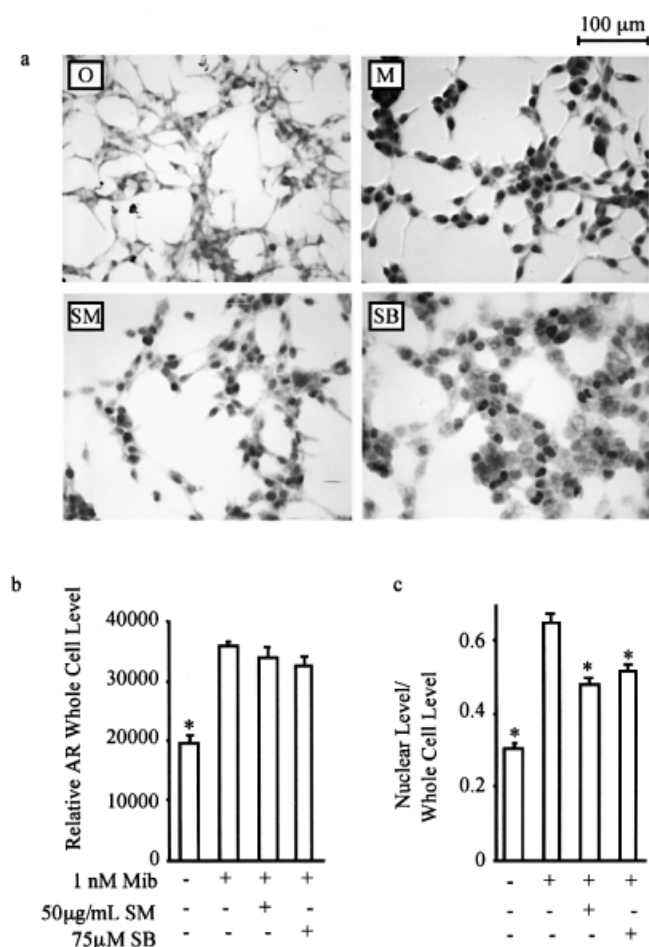
**Fig. 4.** Effects of SM and SB on whole cell AR levels (a), transcription activity of the AR promoter (b) and ligand-binding activity of the AR (c). The upper panel in (a) shows a representative western blot analysis of total AR protein levels in LNCaP cells and Ponceau S staining, representing protein loading. The lower panel in (a) is a densitometric analysis of total AR protein levels from three independent experiments. \* $P < 0.05$ , when compared with the group treated with Mib alone. LNCaP cells were treated with SM and SB in the presence of 1 nM Mib for 24 h in the above analyses. The transcription activities in (b) are expressed as relative light units/unit β-gal. pGL3 denotes a pGL3-Basic luciferase vector as a control.

Previous studies have indicated that reduced AR expression may cause loss of function of the AR (10,12). Therefore, we first checked if SM and SB decrease expression of the AR. As shown in Figure 4a, SM and SB did not affect androgen-enhanced AR protein levels in whole cell extracts ( $P > 0.05$ ). Moreover, when a luciferase reporter plasmid containing the AR promoter was transfected into LNCaP cells, AR promoter activity was not affected by SM ( $P > 0.05$ ) (Figure 4b). This

further supports the hypothesis that SM-mediated inhibition of AR function might not be due to an alteration in total AR protein level. To exclude the possibility that SM or SB might affect the androgen-binding ability of the AR, a ligand-binding assay was performed which showed that SM and SB did not alter androgen-binding activity of the AR ( $P > 0.05$ ) (Figure 4c).

As a transcription factor, nuclear AR level is critical for its function. So AR protein level in the nucleus was further investigated. As seen in Figure 5a and b, both SM and SB significantly reduced nuclear AR levels. Since SM did not affect the nuclear level of transcription factor Sp1 (Figure 5c) and SB is the major component of SM, we may conclude that both SM and SB specifically reduced nuclear AR level. In addition, consistent with the above results, SM seems to exert greater effects (~2-fold) than SB. Hence, SM or SB reduces AR function in LNCaP cells by lowering nuclear AR level.

Immunocytochemical staining was performed to measure the alteration in AR protein levels in the whole cell and



**Fig. 6.** Immunohistochemical staining of the AR. (a) AR staining. O, vehicular control; M, 1 nM Mib; SM, 1 nM Mib + 50 µg/ml SM; SB, 1 nM Mib + 75 µM SB. (b) Bar graph showing whole cell AR levels from (a). (c) The ratio of nuclear AR levels over whole cell AR levels in (a). LNCaP cells were treated with SM and SB in the presence of 1 nM Mib for 24 h and stained for AR with a monoclonal antibody against the human AR. Error bars indicate the standard errors of 20–25 cells in each treatment. \* $P < 0.05$ , when compared with the group treated with Mib alone.

nucleus more directly compared with the above experiments. Again, in the absence of androgens AR protein level was low (Figure 6a). Mib increased AR level significantly and most of the AR was located in the nucleus (Figure 6a). The relative whole cell levels of AR are shown in Figure 6b and the ratio of AR in the nucleus over that in the whole cell is shown in Figure 6c. Consistent with the western blot results shown in Figure 4a, SM and SB did not affect whole cell AR level in the presence of Mib (Figure 6a and b). However, nuclear AR level was significantly reduced by SM and SB treatment ( $P < 0.05$ ) (Figure 6a and c).

## Discussion

In this report we have demonstrated that both SM and SB can block androgen-stimulated proliferation of LNCaP cells. In the presence of androgens, SM and SB inhibit expression of PSA, hK2 and FKBP51, suggesting that SM and SB negatively affect AR-mediated androgen action. Gene transfer experiments using a PSA promoter or an ARE-driven promoter have shown that SM and SB can indeed inhibit AR function. It was also noted that SM at a concentration of 50 µg/ml, containing the

equivalent of 75 µM SB, shows a stronger inhibitory effect on AR function than does SB. It seems to be consistent in most of our studies that SM usually had a stronger effect than SB. This might be due to additional flavonoid components in SM (15).

A previous *in vivo* study showed that androgen withdrawal causes growth arrest of androgen-responsive cancer cells in early G<sub>1</sub> phase with increases in p53, p21/waf1 and p16 and a decrease in cyclin D1, as well as alterations in expression of some other cell cycle-related proteins (16). These changes seem to be correlated with a decrease in AR protein. An *in vitro* study also confirmed that androgens exert an effect on G<sub>1</sub>-S progression in LNCaP cells by affecting the activities or expression levels of multiple G<sub>1</sub> regulatory elements such as cyclin-dependent kinases (CDK) CDK2 and CDK4 and cyclin D3 (17). The exact mechanisms by which the AR affects the cell cycle and cell cycle-related protein activities are not clear, although there is an indication that androgen/AR can directly regulate expression of some cell cycle-related proteins. Nonetheless, these studies all indicate a central role of androgen/AR in prostate cell growth.

Many factors may affect function of the AR, for example protein level, phosphorylation, nuclear localization and ligand-binding of AR, coactivators, heat shock proteins, etc. Our data show that SM and SB reduce the nuclear level of AR but not the whole cell level of AR, indicating that SM- or SB-mediated loss of AR function may be partly due to a reduction in nuclear AR levels. Our study demonstrates that SM and SB have a novel action in interfering with AR function.

We found that FKBP51 is induced by androgens and that this induction was repressed by SM and SB. It is known that FKBP51 is present in several steroid receptor-hsp90 heterocomplexes, including the progesterone and glucocorticoid receptors (18,19). Although the role of FKBP51 in receptor heterocomplexes is not clear, it has been proposed that immunophilins may act as nuclear shuttle proteins to direct and/or facilitate nuclear transportation of steroid receptors (20). If this model is true, reduced FKBP51 may cause a reduction in movement of AR from the cytoplasm to nucleus and thus nuclear AR level would be decreased. Alternatively, it has been reported that ligand-activated AR has a high affinity for the nuclear matrix (21). It is possible that SM and SB reduce the tight interaction between AR and the nuclear matrix, which potentially causes a redistribution of AR between the nucleus and cytoplasm.

Although both western blot analysis and immunocytochemical staining consistently showed a decreased nuclear AR level after SM or SB treatment, nuclear AR level determined by western blot analysis seems to be lower compared with that determined by immunocytochemical staining. One possible explanation is that due to the reduced binding affinity of AR for the nuclear matrix, AR might more easily leak out of nuclei during the mechanical process of nuclear extraction, therefore less AR remained in the nuclear fraction. Also, we cannot rule out other possibilities, for example some specific nuclear membrane alteration that may be involved in SM-mediated AR leakage. Importantly, our novel findings seem to warrant further investigations on the detailed mechanisms of the effect of SM and SB on AR localization.

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