

The antiandrogen bicalutamide activates the androgen receptor (AR) with a mutation in codon 741 through the mitogen activated protein kinase (MARK) pathway in human prostate cancer PC3 cells

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Abstract. The objective of this study was to assess the effect of antiandrogen on the activation of mutated androgen receptor (AR) and its signaling pathway in prostate cancer. We transfected the AR gene with a point mutation at codon 741 (tryptophan to leucine; W741L) into human androgen-independent prostate cancer PC3 cells lacking the expression of AR, and established PC3 cells overexpressing mutant type AR (PC3/W741L). Changes in the phenotype in these cells were compared to those in PC3 cells transfected with wild-type AR (PC3/Wild) and control vector alone (PC3/Co). There was no significant differences in the growth among PC3/Co, PC3/Wild and PC3/W741L cells. A transactivation assay using these cells showed that bicalutamide activated W741L mutant type AR, but not wild-type AR, while hydroxyflutamide failed to activate either type of ARs. Treatment with specific inhibitors of the MAPK or STS3 pathway (UO126 or AG490, respectively), in contrast to treatment with the Akt pathway inhibitor LY294002, significantly inhibited the dihydrotestosterone-induced activation of both wild-type and mutant ARs; however, activation of W741L mutant AR by bicalutamide was significantly inhibited by treatment with UO126, in contrast to treatment with AG490 or LY294002. Furthermore, treatment of PC3/W741L with bicalutamide, in contrast to treatment with hydroxyflutamide, resulted in significant upregulation of phosphorylated p44/42 MAPK. These findings suggest that the MAPK pathway might be involved in the activation of the AR with a point mutation at codon 741 induced by treatment with the antiandrogen bicalutamide.

Introduction

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-specific deaths in men in Western industrialized countries. Despite intensive efforts in the field of prostate cancer research, androgen withdrawal therapy has continued to be the mainstay of treatment for men with advanced prostate cancer since the report by Huggins and Hodges in 1941 (1). In recent years, a novel therapeutic strategy of androgen ablation by combining luteinizing hormone-releasing hormone analog and anti-androgen was introduced as 'maximal androgen blockade (MAB)'. Initially, ~90% of patients with advanced prostate cancer favorably respond to MAB therapy; however, androgen-independent (AI) progression ultimately occurs within a few years in the majority of these patients (1,2). During the course of AI progression, a paradoxical phenomenon, 'antiandrogen withdrawal syndrome (AWS)', which is characterized by the improvement of clinical findings associated with prostate cancer following discontinuation of therapeutic antiandrogen, has been shown to occur in 25-50% of patients treated with androgen ablation therapy (3,4).

Several investigators have reported the activation of some types of mutant androgen receptors (ARs) by anti-androgens (5-9). For example, Fenton *et al* reported that flutamide works as an agonist for T877S and H874Y mutant ARs (6), while Hara *et al* showed the stimulation of W741C and W741L mutant ARs by bicalutamide (8). Considering these findings, AR gene mutations could be associated, at least in part, with the development of AWS; however, little is known about the precise molecular mechanisms that trigger the conversion of an initial inhibitory to subsequent stimulatory effect of antiandrogen on the progression of prostate cancer cells. In this study, we introduced AR gene with a point mutation at codon 741 into human AI prostate cancer PC3 cells lacking the expression of AR, and investigated the mechanism involved in AWS using PC3 cells overexpressing this type of mutated AR.

Materials and methods

PC3, derived from human prostate cancer, was purchased from the American Type Culture Collection (Rockville, MD).

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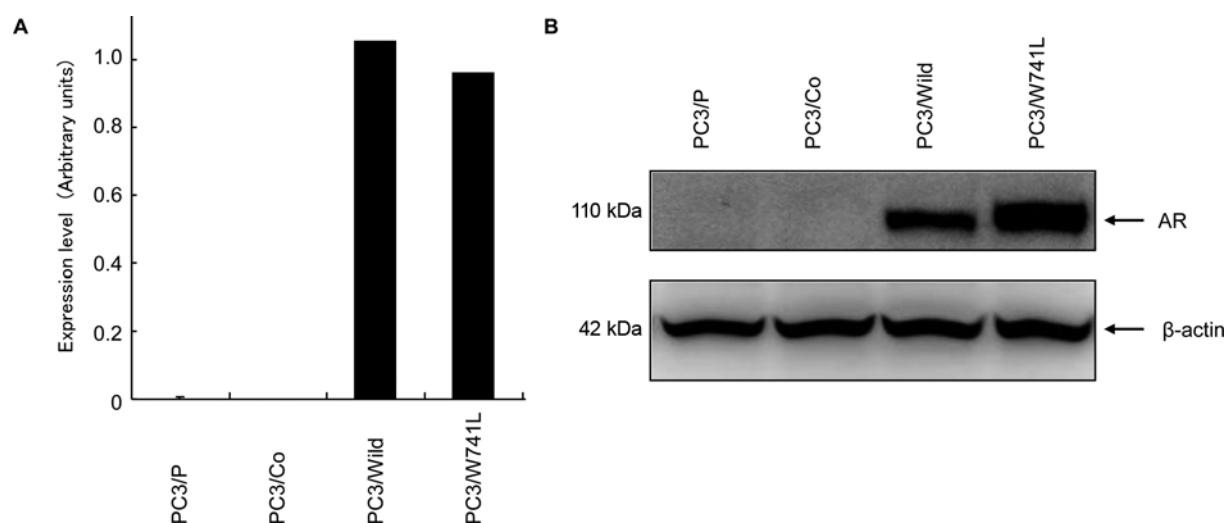


Figure 1. Expression levels of androgen receptor (AR) in PC3 sublines (PC3/P, parental cell line of PC3; PC3/Co, control vector-only transfected cell line; PC3/Wild; wild-type AR-transfected cell line, and PC3/W741L, W741L mutant type AR-transfected cell line). (A) Real-time RT-PCR was performed to analyze the expression levels of AR mRNA in each cell line in three independent experiments. Bars, SD. (B) Western blotting was performed to analyze the expression levels of AR protein in each cell line.

Cells were maintained in RPMI (Life Technologies, Gaithersburg, MD) supplemented with 5% heat-inactivated fetal bovine serum.

The cDNA fragments encoding human wild-type AR or human AR with a point mutation at codon 741 (tryptophan to leucine; W741L) were inserted into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). Purified pcDNA3.1 alone (as a control) or pcDNA3.1 with wild-type or W741L mutant type AR was transfected into PC3 cells by the liposome-mediated gene transfer method as previously described (10).

Total RNA (1 μ g) extracted from each cell line was reverse transcribed using an Oligo dT and Superscript pre-amplification system (Life Technologies). Real-time RT-PCR was performed using a Sequence Detector (ABI PRISM 7700, PE Applied Biosystems, Foster City, CA) based on the TaqMan assay according to the manufacturer's instructions as described previously (11). All specimens were analyzed in triplicate and the mean values were used for quantification. The quantification value of AR mRNA was described relative to β -actin mRNA.

Western blot analysis was performed as previously described (10). Briefly, samples containing equal amounts of protein (15 μ g) from lysates of the cultured PC3 sublines were electrophoresed on an SDS-polyacrylamide gel, transferred to a nitrocellulose filter, and then incubated for 1 h with an anti-human AR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), β -actin monoclonal antibody (Santa Cruz Biotechnology), total or phosphorylated p44/42 mitogen-activated protein kinase (MAPK) antibody (Cell Signaling Technology, Danvers, MA). The filters were subsequently incubated for 30 min with horseradish peroxidase-conjugated secondary antibodies, and specific proteins were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Arlington Heights, IL).

The effects of dihydrotestosterone (DHT), bicalutamide or hydroxyflutamide (Sigma-Aldrich, Tokyo, Japan) on the

growth of each cell line were assessed using MTT (Sigma) as described previously (10). Briefly, 1×10^4 cells were seeded into 96-well microtiter plates, and then treated with indicated concentrations of DHT, bicalutamide or hydroxyflutamide. After 48 h of incubation, 5 mg/ml MTT was added to each well, followed by incubation for 4 h at 37°C. The formazan crystals were dissolved in DMSO. The optical density was determined with a microculture plate reader (Becton Dickinson Labware, Lincoln Park, NJA) at 540 nm.

Transactivation assays were performed as described previously (9). Briefly, PC-3 sublines were seeded in each well of 24-well plates in phenol red-free RPMI-1640 and were transiently cotransfected with 500 ng of p5.3PSAp-Luc and 5 ng of pTK-RL using lipofectamine (Life Technologies). Luciferase activity of the cell lysate in the indicated concentration of DHT, bicalutamide, hydroxyflutamide, UO126, LY294002 and/or AG490 (SA Bioscience, Frederick, MD) was measured using Dual-Luciferase reporter assay system (Promega, Madison, WI) with a multiplate luminometer (MicroLumat Plus LB96V, Berthold Technologies, Bad Wildbad, Germany). The results are presented as the fold induction that is the relative luciferase activity of the treated cells divided by that of the control. All the experiments were carried out in triplicate.

All outcomes are presented as the mean \pm SD. Differences were analyzed using the unpaired t-test, and $p < 0.05$ was considered to indicate significant difference.

Results

PC3 cells were transfected with the expression vector alone or that containing wild-type AR or W741L mutant type AR cDNA. After drug selection, several independent clones were established. Real-time RT-PCR and Western blotting were subsequently performed to evaluate the expression levels of AR mRNA and protein, respectively, in PC3 sublines. As shown in Fig. 1, neither parental PC3 (PC3/P) nor control vector only-transfected (PC3/Co) cell lines express a detectable

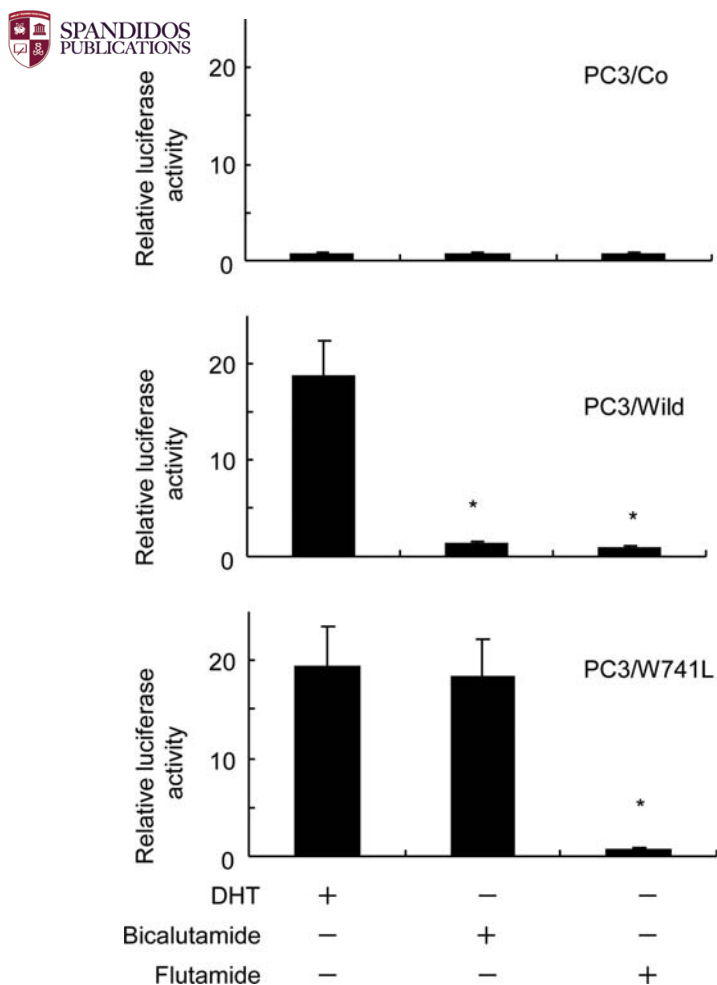


Figure 2. Transcriptional response of AR to dihydrotestosterone (DHT), bicalutamide or hydroxyflutamide in PC3 sublines (PC3/Co, control vector-only transfected cell line; PC3/Wild; wild-type AR-transfected cell line, and PC3/W741L; W741L mutant type AR-transfected cell line). PC3 sublines were treated with 100 nM DHT, 1 μ M bicalutamide or 1 μ M hydroxyflutamide, and luciferase activity was measured 48 h after drug treatment. Bars, SD; *differs from activity after treatment with DHT ($p < 0.01$).

level of AR on either assay, whereas abundant levels of AR expression were observed in wild-type AR-transfected cell line (PC3/Wild) and mutant type AR-transfected cell line (PC3/W741L) at both the mRNA and protein levels. In the following experiments, almost identical findings were obtained from PC3/P and PC3/Co; therefore, we hereafter present the data for PC3/Co, PC3/Wild and PC3/W741L only.

We initially compared the growth of PC3 sublines in culture supernatants with and without DHT, bicalutamide and hydroxyflutamide. There were no significant differences in the growth of PC3/Co, PC3/Wild and PC3/W741L cultured in serum-free medium. Furthermore, the growth patterns of these sublines were not significantly influenced by additional treatment with 100 nM DHT, 1 μ M bicalutamide or 1 μ M hydroxyflutamide (data not shown).

Because of the lack of differences in the growth of PC3 sublines under several conditions, it was then investigated by transactivation assays whether differential transcriptional responses were different to DHT, bicalutamide and

hydroxyflutamide according to the types of ARs. As shown in Fig. 2, there was no luciferase activity detected in PC3/Co after treatment with any agent; while DHT activated both wild-type and W741L mutant type ARs. In addition, there was no significant effect of hydroxyflutamide on the induction of luciferase activity in PC3/Wild and PC3/W741L, while W741L mutant type AR, but not wild-type AR, was aberrantly stimulated by bicalutamide.

To characterize the involvement of signal transduction pathways in the activation of ARs by several agents, changes in luciferase activities after additional treatment of PC3 sublines with UO126, LY294002 or AG490, a selective inhibitor of MAPK, phosphoinositol 3'-kinase (PI3K)/Akt or Janus-activated kinase (JAK)/signal transducers and activation of transcription (STAT) pathway, respectively, were examined. As shown in Fig. 3A, luciferase activities induced by DHT in both PC3/Wild and PC3/W741L were significantly suppressed by treatment with either UO126 or AG490, but not that with LY294002. Furthermore, despite the lack of significant difference in the transcriptional responses of wild-type AR to UO126, LY294002 or AG490, W741L mutant type AR by bicalutamide was markedly inhibited by treatment with UO126, but not that with LY294002 or AG490 (Fig. 3B).

To assess whether treatment of PC3 sublines with antiandrogens influences MAPK pathways, Western blot analyses of both phosphorylated and total MAPK were performed. As shown in Fig. 4, phosphorylated MAPK could not be detected in PC3/Co treated with and without DHT, bicalutamide and hydroxyflutamide. In PC3/Wild, DHT slightly, but not significantly, induced the phosphorylation of MAPK; however, the expression level of phosphorylated p44/42 MAPK was not significantly changed by treatment with either bicalutamide or hydroxyflutamide. Treatment of PC3/W741L with DHT or bicalutamide, but not that with hydroxyflutamide, resulted in the significant upregulation of phosphorylated p44/42 MAPK.

Discussion

In 1993, Scher and Kelly first described AWS, that is the phenomenon of improvement in subjective and objective findings following discontinuation of antiandrogen flutamide in a subset of prostate cancer patients treated with androgen ablation therapy (12). Thereafter, this phenomenon has been widely observed in several kinds of antiandrogens (3,4), and the concept of 'alternative antiandrogen therapy' has been developed based on these findings; that is, it has been shown to be useful to use other antiandrogens for patients whose disease becomes refractory to primary MAB (3,13,14). Although the detailed molecular mechanisms underlying AWS remain largely unknown, some types of mutated AR have been suggested to be involved in AWS (5-9). Of several mutated ARs, W741L in addition to W741C, which locate in the ligand-binding domain of AR gene, were shown to be activated by bicalutamide (8). In this study, we established human AI prostate cancer PC3 cells over-expressing wild-type AR and W741L mutant type AR, and compared the effects of bicalutamide, which has become the most prevalent pure antiandrogen, between these cell lines,

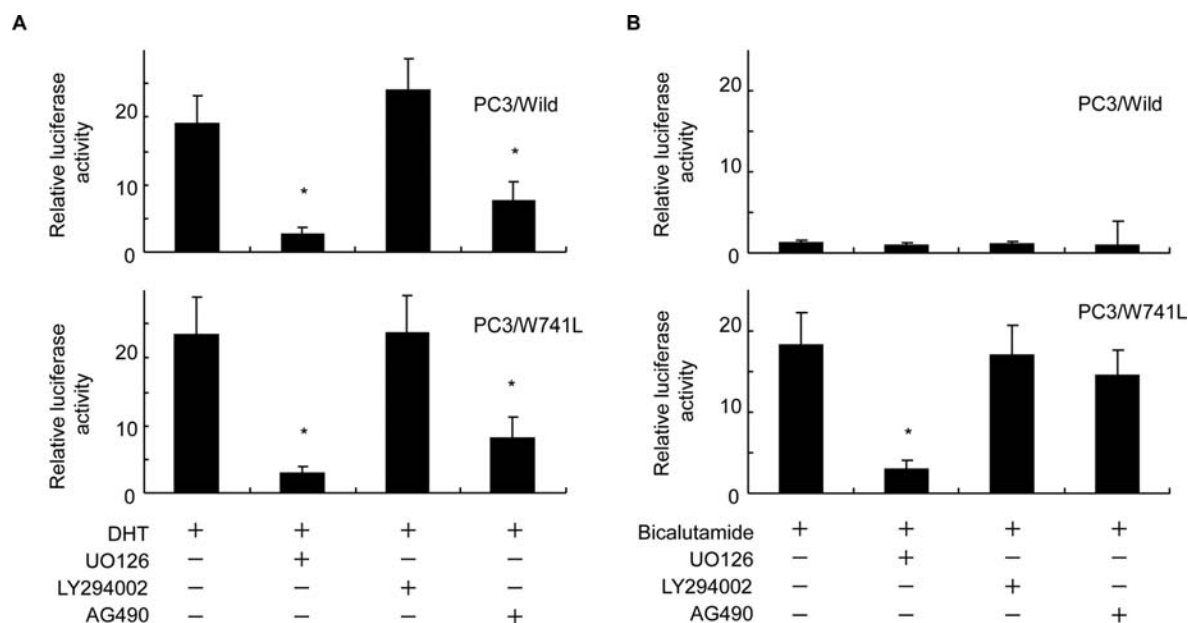


Figure 3. (A) Transcriptional response of AR to dihydrotestosterone (DHT) in combination with UO126, LY294002 or AG490 in PC3 sublines (PC3/Wild; wild-type AR-transfected cell line, and PC3/W741L; W741L mutant type AR-transfected cell line). PC3 sublines were treated with 100 nM DHT either alone or in combination with 5 μ M UO126, LY294002 or AG490, and luciferase activity was measured 48 h after drug treatment. Bars, SD; *differs from activity after treatment with DHT alone ($p<0.01$). (B) Transcriptional response of AR to bicalutamide in combination with UO126, LY294002 or AG490 in PC3 sublines (PC3/Wild; wild-type AR-transfected cell line, and PC3/W741L; W741L mutant type AR-transfected cell line). PC3 sublines were treated with 1 μ M bicalutamide either alone or in combination with 5 μ M UO126, LY294002 or AG490, and luciferase activity was measured 48 h after drug treatment. Bars, SD; *differs from activity after treatment with DHT alone ($p<0.01$).

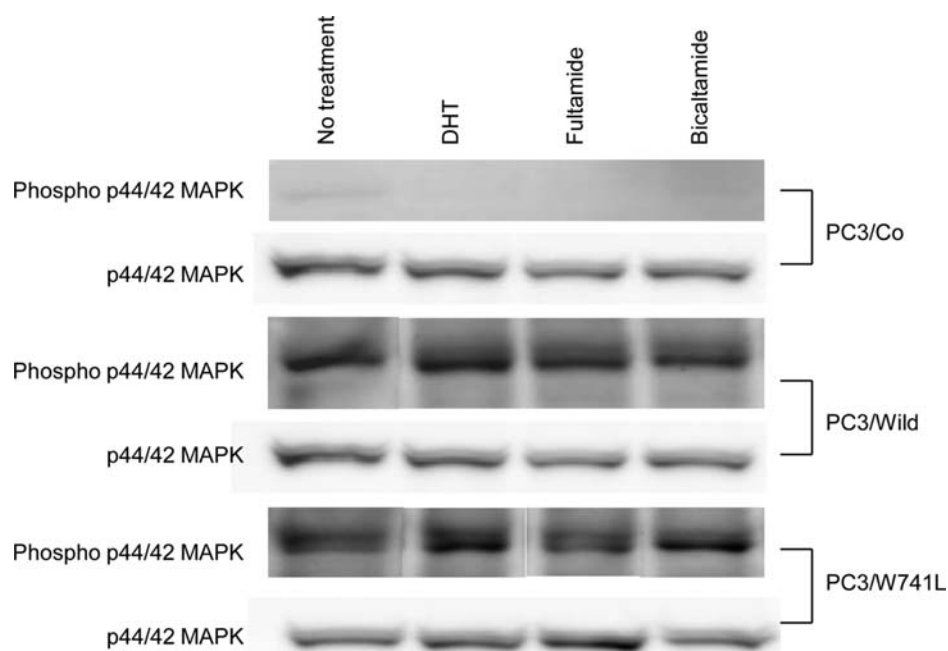


Figure 4. Effects of treatment of PC3 sublines (PC3/Co, control vector-only transfected cell line; PC3/Wild; wild-type AR-transfected cell line, and PC3/W741L; W741L mutant type AR-transfected cell line) with dihydrotestosterone (DHT), bicalutamide or hydroxyflutamide on their mitogen-activated protein kinase (MAPK) pathways. PC3 sublines were treated with 100 nM DHT, 1 μ M bicalutamide or 1 μ M hydroxyflutamide, and proteins were extracted from each cell line 48 h after drug treatment. Both phosphorylated and total p44/42 MAPK protein levels were analyzed by Western blotting.

in order to elucidate the mechanism of bicalutamide withdrawal syndrome.

In this study, we initially showed that there were no significant differences in the growth patterns between wild-type and W741L mutant type AR-transfected PC3 sublines cultured in the media with and without DHT, bicalutamide and hydroxyflutamide. As shown in different systems in previous studies (8,9), bicalutamide, but not hydroxyflutamide, could act as an agonist for W741L mutant type AR; however, wild-type AR was not activated by either bicalutamide or



lutamide. These findings suggest that the model used in this study would be suitable for investigating the transcriptional responses of ARs to antiandrogens.

The phenotype of prostate cancer, particularly that during progression to androgen independence, has been shown to be regulated by signal transduction through AR (15); accordingly, it was then examined whether the major signal transduction pathways via AR, including MAPK, PI3K/Akt and JAK/STAT pathways, are involved in the activation of AR by antiandrogens. Although transcriptional responses of both wild-type and W741L mutant type ARs by DHT were inhibited by selective inhibitor of either MAPK or JAK/STAT pathway, but not that of PI3K/Akt pathway, the inhibitor of MAPK markedly suppressed the bicalutamide-induced activation of W741L mutant type AR, despite the lack of significant effects of the inhibitors of PI3K/Akt and JAK/STAT pathways. Furthermore, treatment of W741L mutant type AR-transfected PC3 cells with bicalutamide induced significant upregulation of phosphorylated p44/42 MAPK. Considering these findings, bicalutamide may exert agonistic activity in prostate cancer cells with W741L mutant type of AR during androgen ablation therapy through the activation of MAPK pathway.

A few investigators have analyzed mechanisms underlying AWS other than mutations of AR gene (16-18). Pitkänen-Arsiöla *et al* reported that stabilization of AR and its co-regulator, DJ-1, in the absence of androgen may partially account for AWS (16). Lee *et al* described the possible mechanism to explain hydroxyflutamide withdrawal syndrome; that is, when prostate cancer acquires an AI phenotype, the continuous administration of hydroxyflutamide triggers the activation of MAPK pathway without activation of AR, resulting in the stimulation of prostate cancer progression (17). Bohl *et al* suggested the impact of structural modifications through the binding to bicalutamide to W741L mutant type of AR on bicalutamide withdrawal syndrome (18). Collectively, these findings suggest that AWS is the phenomenon consisting of a complex process involving the molecular interaction among several molecules, including co-regulators of AR and signal transducers via AR in addition to antiandrogens and ARs.

In conclusion, treatment of PC3 cells harboring AR with a point mutation at codon 741 with bicalutamide resulted in the marked activation of this type of mutant AR through the signal transduction pathway of MAPK. Therefore, from a clinically relevant perspective, it would be of interest to further investigate the pathway mediating transcriptional activation of AR according to the combination between antiandrogen and mutated AR to help clarify the molecular mechanisms underlying AWS.

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