Studies on the Inhibition of Androgen Receptor Nuclear Translocation in the Castration-resistant Prostate Cancer Cells

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Contents

Abstract

Castration resistance is a lethal problem for advanced prostate cancer patients. The major mechanisms of such resistance are androgen receptor (AR) overexpression, AR mutation, and androgen-independent activation of AR. The aim of the present study was to examine the influence of inhibiting AR nuclear transfer, a process common to these three major mechanisms. First, I established a castration-resistant prostate cancer (CRPC) cell line with androgen-independent activation of AR, which is the first such cell model for studying this mechanism that has been proposed but not yet experimentally proven. LNCaP-CS10 was established from a hormone-sensitive prostate cancer (HSPC) cell line, LNCaP, by treatment under an androgen-depleted condition with 10 μM bicalutamide. Compared to LNCaP cells, LNCaP-CS10 cells did not show AR overexpression or any additional AR mutation. LNCaP-CS10 cells exhibited an increase in proliferation and prostate-specific antigen (PSA) production in the absence of androgen, and induction of these phenomena was observed following treatment with bicalutamide. In the absence of androgen, AR knockdown suppressed LNCaP-CS10 proliferation and PSA production. AR nuclear translocation was induced by bicalutamide treatment to LNCaP-CS10 cells. The growth and PSA production of xenografted LNCaP-CS10 tumors were not inhibited by castration or administration of bicalutamide. Next, to examine the influence of inhibiting AR nuclear transfer, I used an AR nuclear translocation-inhibiting compound, CH5137291, which my coworkers and I developed recently, and compared its activity and characteristics with those of bicalutamide. I analyzed three cell lines corresponding to the three mechanisms of castration resistance: LNCaP-BC2, showing AR overexpression; PC3, harboring mutant AR through transient transfection; and LNCaP-CS1, showing androgen-independent AR activation. Two other cell lines, VCaP and LNCaP, were also used as HSPC cells. An *in vitro* intracellular localization assay showed that CH5137291 inhibited the nuclear translocation of wild-type ARs as well as W741C and T877A mutant ARs. In addition, CH5137291 exhibited a complete AR antagonist effect without an AR agonist effect on the transcriptional activity of these types of ARs. In contrast, bicalutamide did not inhibit the nuclear translocation of these ARs, and showed a partial or full agonist effect on transcriptional activity. CH5137291 inhibited cell growth more strongly than bicalutamide in VCaP and LNCaP cells as well as in LNCaP-BC2 and LNCaP-CS10 cells *in vitro*. In xenograft models, CH5137291 strongly inhibited the tumor growth of LNCaP, LNCaP-BC2, and LNCaP-CS10 cells, whereas bicalutamide showed a weaker

effect on LNCaP and almost no effect on LNCaP-BC2 and LNCaP-CS10 xenografts. The plasma levels of PSA correlated well with the antitumor effect of both agents. CH5137291 inhibited the growth of LNCaP tumors that had become resistant to bicalutamide treatment. A docking model suggested that CH5137291 intensively collided with the M895 residue of helix 12, and therefore strongly inhibited the folding of helix 12, a cause of AR agonist activity, in wild-type and W741C-mutant ARs. In normal cynomolgus monkeys, the serum concentration of CH5137291 increased dose-dependently and the PSA level decreased by 80% when CH5137291 dose was 100 mg/kg. Overall, in the present study, I clearly demonstrated that castration resistance could be acquired by androgen-independent AR activation as well as by AR overexpression or AR mutation, and that inhibition of AR nuclear translocation could offer a novel therapeutic approach against the major types of CRPCs and prolong the response duration against HSPC.

General introduction

Prostate cancer is the most common epithelial cancer and the second leading cause of cancer-related deaths in males in the United States (1-5). Because most prostate cancers are initially dependent on androgens for their growth, patients with prostate cancer receive androgen depletion therapies. Existing androgen depletion therapies are initially effective for most patients against this disease (6,7). At this time, prostate cancer is referred to as hormone-sensitive prostate cancer (HSPC). However, the cancer often acquires resistance and becomes refractory to these therapies in several years (castration-resistant prostate cancer, CRPC), which is a major clinical challenge (8,9). Because the expression of prostate-specific antigen (PSA), a target gene of androgen receptor (AR) and a tumor marker reflecting the proliferation of prostate cancer, is observed in the majority of CRPC cases, the AR-dependent growth signal is thought to remain in CRPC (8-10). Once HSPC becomes CRPC, there are few effective treatments, and thus novel treatment methods are required.

Androgen refers to all male hormones, including testosterone. Testosterone is mainly synthesized in the testis and partly in the adrenal gland, which account for approximately 95% and 5% of total androgen, respectively. AR has very high sequence similarity with other nuclear receptors (e.g., progesterone receptor, PR; glucocorticoid receptor, GR; mineralocorticoid receptor, MR; and estrogen receptors, ERs) belonging to the nuclear receptor superfamily. Steroid hormone receptors belonging to the nuclear receptor superfamily generally consist of three structural units: AF-1, which is a ligand-independent transcriptional activation region at the N-terminus; a DNA-binding domain; and AF-2, which contains a ligand-binding domain and exists at the C-terminus showing ligand-dependent transcriptional activity (6). Helix 12 of AR takes on a three-dimensional structure like a lid in a pocket-like space to which a ligand binds. In the unliganded state, AR exhibits an unfolded structure such that the lid is opened, and when the ligand is bound, helix 12 takes on a folded structure in which the lid is closed. The androgen antagonists bind to the ligand-binding domain competitively with the ligand, thereby interfering with the folding the helix 12 and ultimately inhibiting transcriptional activity (11,12).

Here, I will provide a brief overview of the AR signal, which is the key to prostate cancer proliferation. Testosterone secreted from the testis and adrenal glands is metabolized to dihydroxytestosterone (DHT), activated androgen, by 5α reductase in prostate cancer cells (13). Testosterone secretion from the testis occurs through

stimulation of luteinizing hormone-releasing hormone (LH-RH) from the hypothalamus and luteinizing hormone (LH) from the pituitary gland. AR, to which DHT binds as a ligand, forms a dimer, transitions from the cytoplasm to the nucleus, and shows transcriptional activity via binding to the androgen response element on DNA as a transcription factor (13,14).

Androgen depletion therapies include surgical castration, treatment with an LH-RH agonist aimed at inhibiting testicular-derived androgen secretion, and treatment with an AR antagonist intended to inhibit the action of androgens (6,7). An agonist is a substance that binds to and activates the receptor, whereas an antagonist is a substance that binds to and inhibits the activity of the receptor. The LH-RH agonist inhibits the secretion of testosterone from the testes by decreasing the expression of the pituitary LH-RH receptor upon its continuous administration. However, this cannot suppress the secretion of androgen from the adrenal glands. Therefore, AR antagonists such as bicalutamide and flutamide, which inhibit the binding of androgen and AR, are used in combination with an LH-RH agonist (7). This combination therapy is called total androgen blockade (6,7). However, these therapies also cannot completely block the AR signal. Therefore, resistance is ultimately acquired in most patients (8).

There are three main mechanisms of castration resistance reported thus far (13). The first mechanism is overexpression of AR, which increases the sensitivity to androgen, resulting in accelerated nuclear translocation of AR and proliferation even at androgen concentrations that are kept low by androgen depletion therapy and where proliferation is not observed in HSPC cells (15-17). The second mechanism is mutation of AR. Mutations in the ligand-binding domain of AR create loss of ligand specificity, so that anti-androgens act as agonists for the mutated ARs (18-22). In particular, the W741C and T877A mutant ARs are reported to be activated by bicalutamide and flutamide, respectively (22). Many models have been established for studying these two mechanisms, with extensive research conducted to date (15,17-19,21,22). The third mechanism is proposed as androgen-independent AR activation. However, there is no available model for studying this mechanism, and thus it has not yet been clearly proven. The frequency of the clinical occurrence of these three mechanisms is 25–30%, 10–30%, and ~30%, respectively.

AR nuclear translocation is a common signaling pathway not only for HSPC but also for CRPC. Therefore, I hypothesized that inhibition of AR nuclear translocation would inhibit the proliferation of CRPC cells via the three resistance mechanisms described above. The aims of this study were (I) to establish a CRPC cell line with characteristics of androgen-independent AR activation, a model that had not been established when I

started this study (Chapter I); and (II) to examine the effect of inhibition of AR nuclear translocation, a common signaling pathway of CRPC, on CRPC cell lines with androgen-independent activation of AR, overexpression of AR, and with mutations in AR genes (Chapter II).

Chapter I.

Establishment and characterization of an AR-dependent, androgen-independent human prostate cancer cell line, LNCaP-CS10

Introduction

Recent analysis revealed that expression of AR remained in most castration-resistant prostate cancer (CRPC) specimens and these patients were still PSA positive (8-10). These observations suggest that the AR signal is still active in castration-resistant prostate cancer cells, and therefore AR is a potential therapeutic target for castration-resistant prostate cancer.

In a recent review, Pienta et al. (Fig. 1) (13) suggested three major mechanisms of androgen refractoriness: i) overexpression of AR, ii) mutations in AR genes and iii) androgen-independent activation of AR. AR overexpression leads to hypersensitivity to very low levels of androgen in patients treated with hormonal therapies (15-17). Mutations in the ligand-binding domain of AR create loss of ligand specificity and so anti-androgens act as agonists for the mutated ARs (18-22). The androgen-refractory cells with androgen-independent activation of AR are termed “outlaw”. In outlaw tumors, ARs are activated by androgen-independent pathways, such as deregulated cytokines and growth factors, including IL-6, IGF and EGF (23-29).

Although several castration-resistant prostate cancer cell lines with AR overexpression or AR mutation have been established, no castration-resistant prostate cancer cell lines using the outlaw pathway have been established (17,22,30-32).

In this study, I isolated a novel bicalutamide-resistant and androgen-independent prostate cancer cell line (LNCaP-CS10) and demonstrated its outlaw pathway both *in vitro* and *in vivo*.

Material and methods

Cell culture

The androgen-dependent human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in 10% FCS medium consisting of RPMI1640 (Sigma, St. Louis, MO, USA) and 10% FCS (Japan Bioserum, Hiroshima, Japan) at 37°C in 5% CO_2. The LNCaP-CS10 cells were maintained in 10% DCC medium consisting of phenol red-free RPMI1640 (Invitrogen, Carlsbad, CA, USA) and 10% charcoal/dextran-treated FCS (DCC-FCS) (Hyclone Laboratories, Logan, UT, USA) in the presence of 10 μM bicalutamide.

Cell proliferation assays

Androgen depleted cells were prepared by preincubation in 10% DCC medium for 3 days and the cells were then plated onto a 96-well poly-D-lysine coated plate (Beckton Dickinson, Franklin Lakes, NJ, USA) at 1×10^4 cells/well (LNCaP) or 5×10^3 cells/well (LNCaP-CS10) in 10% DCC medium. After overnight incubation at 37°C in 5% CO_2, R1881 (NEN Life Science, Boston, MA, USA) or bicalutamide was added. After a further 9 days of incubation at 37°C in 5% CO_2, the number of cells remaining was counted by DNA quantity assay (FluoReporter Blue Fluorometric dsDNA Quantitation Kit; Invitrogen).

Quantification of cellular PSA

After androgen depletion for 3 days, the cells were plated onto a 24-well poly-D-lysine coated plate (Beckton Dickinson) at 2×10^5 cells/well in 10% DCC medium, incubated overnight and R1881 or bicalutamide were added. After 2 days of incubation, the cells were lysed with ice-chilled 1× cell lysis buffer (Cell Signaling) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Basel, Swiss). After centrifugation at 14000 rpm for 10 min at 4ºC, PSA in the supernatant was quantified using PSA ELISA assay (E-plate Eiken PSA; Eiken Chemical, Tokyo, Japan).

Knock-down experiments with AR siRNA

LNCaP and LNCaP-CS10 cells were transfected with AR siRNA (Santa Cruz Biotechnology) or scramble siRNA (Invitrogen) using Hyperfect (Qiagen, Hilden, Germany) and then plated onto a 96-well poly-D-lysine coated plate at 5×10^3 cells/well in 10% FCS medium (LNCaP) or 10% DCC medium (LNCaP-CS10),

respectively. At 6 h, 3 days and 6 days post-transfection, the cell counts were quantified as described above. In order to confirm AR knock-down in the transfected cells, the cells were plated onto a 24-well poly-D-lysine coated plate at 4×10^4 cells/well; intracellular PSA and AR were quantified by AR ELISA assay (Active Motif, Carlsbad, CA, USA) after 3 days.

Preparation of nuclear, cytoplasmic and total fractions

Cells were plated onto 6-well poly-D-lysine coated plates (Beckton Dickinson) at 3×10^5 cells/well in 10% DCC medium. After 3 days of incubation, 10 μM bicalutamide was added and the plates were incubated for another 24 h. Then nuclear and cytoplasmic fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA). Total fraction was prepared as described above. AR was detected by western blot analysis using antibody to AR (N-20; Santa Cruz Biotechnology) and an ECL+ western blotting analysis system (Amersham, Little Chalfont, UK). Immunodetections of c-Jun and IκBα (H-79 and C-15, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the loading control of nuclear and cytoplasmic/total fraction.

Animal experiments

Five-week-old male C.B-17/Icr-scid Jcl severe combined immune-deficient (SCID) mice purchased from CLEA Japan (Tokyo, Japan) were maintained on a 12/12 hour light/dark cycle (lights on at 7 a.m.) with constant temperature (20–26°C) and humidity (35–75%). Food and water were available *ad libitum*. The health of the animals was monitored by daily observation. The protocols of the animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd., and all animal experiments were performed in accordance with the Guidelines for the Accommodation and Care of Laboratory Animals promulgated in Chugai Pharmaceutical Co., Ltd. For xenograft experiments, 2×10^6 cells of LNCaP or LNCaP-CS10 were subcutaneously inoculated with 50% phenol red-free Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) into the flank region of intact or surgically castrated mice. The tumor size was measured by caliper and expressed as cubic millimeters using the formula $0.5 \times length \times width^2$. Tumorigenicity was evaluated according to the number of mice bearing palpable tumors (>50mm^3). Sensitivity of LNCaP or LNCaP-CS10 xenograft models to bicalutamide was evaluated as tumor growth inhibition. When the tumor size reached 110–370 mm^3 (LNCaP) or 90–270 mm^3 (LNCaP-CS10), the animals were randomized and orally administered

either bicalutamide (10 or 100 mg/kg) or the drug vehicle (5% gum arabic). The drug was administered in 4–5 cycles of 5 days on, 2 days off, and tumor size and plasma PSA levels were measured at the designated time points.

Results

Establishment of the androgen-independent, bicalutamide-resistant LNCaP-CS10 cell line.

To establish a total androgen blockade therapy-resistant prostate cancer cell line with outlaw pathway activation, LNCaP cells were incubated in 10% DCC medium in the presence of 10 μM bicalutamide. After a 4-month incubation, the surviving cells in the androgen-depleted media in the presence of bicalutamide were selected and designated as LNCaP-CS10.

LNCaP-CS10 exhibited faster growth and higher PSA production in the androgen-depleted medium compared with LNCaP (4.9- and 8.3-fold, respectively, Fig. 2A and B). The growth of LNCaP-CS10 in the androgen-depleted medium was comparable to that of parental LNCaP cells in the presence of 0.1 nM R1881. Although LNCaP-CS10 grew in an androgen-independent manner, R1881 treatment further stimulated the cell growth and PSA production of LNCaP-CS10 (Fig. 2A and B). Bicalutamide showed agonist activity and induced cell growth and PSA production in a dose-dependent manner in LNCaP-CS10 cells but did not stimulate growth or PSA production of LNCaP cells at any concentration (Fig. 2C and D).

Characterization of the outlaw pathway of CS10 in vitro

To confirm the outlaw mechanism of LNCaP-CS10, I first analyzed the sequence of AR cDNA in LNCaP-CS10 by a direct sequence method using PCR amplified AR cDNA. However, LNCaP-CS10 cells did not express a new alteration of AR sequence in comparison to LNCaP (data not shown). Next, I examined the level of AR expression and subcellular localization in LNCaP-CS10 cells and the effect from bicalutamide. AR protein expression in LNCaP-CS10 was similar to that in LNCaP (Fig. 2E). The bicalutamide treatment did not affect the total or cytoplasmic AR protein level in either LNCaP or LNCaP-CS10 cells (Fig. 2E, data not shown). On the other hand, the level of nuclear AR in the LNCaP-CS10 cells was increased by bicalutamide but not in the LNCaP cells (Fig. 2E).

To examine the involvement of the AR signal on the androgen independence of LNCaP-CS10 cells, AR was suppressed with AR-specific siRNAs. The knockdown effect of these AR siRNAs was confirmed by measuring AR protein levels with ELISA (Fig. 3A and B). The siRNAs inhibited the growth and PSA production of LNCaP cells in the 10% FCS medium (Fig. 3C and E). And, in the androgen-depleted medium, the

AR siRNAs also inhibited the growth and PSA production of LNCaP-CS10 cells (Fig. 3D and F).

I examined the phosphorylation status of AKT and ERK and the protein level of IL-6Rα and performed a comparative genomic hybridization analysis and gene-expression profiling of *in vitro*-cultured LNCaP-CS10. No apparent differences between LNCaP-CS10 and LNCaP were observed for pAKT and pERK protein levels or copy number changes in PTEN genes, but overexpression of the IL-6Rα gene and protein were found in LNCaP-CS10 (data not shown). To investigate whether IL-6 axis activation plays a role in the mechanism of outlaw activation in LNCaP-CS10, I analyzed the effect of anti-IL-6 neutralizing antibody and anti-IL-6Rα neutralizing antibody on the growth of LNCaP-CS10 in the absence of androgen. I found that the cell growth of LNCaP-CS10 was not affected by anti-IL-6 antibody or anti-IL-6Rα antibody in the absence of androgen (data not shown).

Lastly, to analyze the expression of downstream factors for AR signaling in LNCaP-CS10 cells, the levels of gene expression of cyclin-dependent kinase (CDK-1), CDK-2, CDK-4, cyclin A2 and cyclin B1 were examined. No apparent differences between LNCaP-CS10 and LNCaP were found for these factors (data not shown).

Tumorigenicity and bicalutamide resistance of LNCaP-CS10 cells in castrated SCID mice.

In order to confirm the outlaw mechanism of LNCaP-CS10 *in vivo*, I inoculated castrated and non-castrated SCID mice with LNCaP-CS10 and the parental LNCaP cells. In the LNCaP-CS10-inoculated mice, palpable tumors were detected in 90% (9/10) of non-castrated mice and 90% (9/10) of castrated mice 29 days after tumor inoculation (Fig. 4B). In contrast, palpable LNCaP tumors were detected in 80% (8/10) of non-castrated mice and no tumors were found in castrated mice 29 days after tumor inoculation (Fig. 4A). In addition, the growth rate of LNCaP-CS10 tumors was similar in both castrated and non-castrated mice, whereas the growth rate of LNCaP was lower in castrated mice than in non-castrated mice (Fig. 4C and D).

To confirm the bicalutamide resistance of LNCaP-CS10 cells *in vivo*, I administered bicalutamide to the LNCaP-CS10 xenograft model in castrated SCID mice. The tumor growth of LNCaP-CS10 was not inhibited by 10 or 100 mg/kg bicalutamide in the castrated mice (Fig. 5B). The serum PSA level in LNCaP-CS10 tumors was slightly increased by the administration of 10 or 100 mg/kg bicalutamide (Fig. 5D). On the other hand, in non-castrated mice, the growth and the serum PSA level of parental LNCaP

were inhibited to a castration level by the administration of 10 or 100 mg/kg bicalutamide (Fig. 5A and C).

Discussion

I have established an “outlaw” cell line, LNCaP-CS10 that is androgen-independent and AR-dependent from androgen-dependent LNCaP cells after a 4-month incubation in an androgen-depleted medium supplemented with bicalutamide. The androgen independence of LNCaP-CS10 was confirmed by its growth under androgen-depleted conditions. The parent LNCaP cell line showed no growth under the same conditions (Fig. 2A). Furthermore, LNCaP-CS10 produced PSA without androgen (Fig. 2B), indicating that the AR signal was still active.

According to recent studies, the AR signal plays a pivotal role in a large segment of castration-resistant prostate cancers (8-10). The mechanisms of castration resistance include AR overexpression, AR mutation and ligand-independent activation of AR coined the outlaw pathway (13). To understand the mechanism for castration resistance of LNCaP-CS10, I analyzed the level of AR expression and the sequence of AR cDNA. LNCaP-CS10 did not show overexpression of AR or a new alteration of the AR sequence in comparison to LNCaP (Fig. 2E, data not shown). However, the experiments with AR siRNAs revealed outlaw activation that stimulated the growth and PSA production of LNCaP-CS10 cells (Fig. 3D and F).

My tumorigenicity study results demonstrate that LNCaP-CS10 cells formed palpable tumors not only in non-castrated SCID mice but also in castrated SCID mice. The high tumor take rate and the ability to grow in castrated mice indicate that the LNCaP-CS10 cell line is suitable for evaluating new therapeutics for castration-resistant prostate cancer (Fig. 4B and D).

To determine whether LNCaP-CS10 cells show bicalutamide resistance *in vivo*, I inoculated castrated SCID mice with LNCaP-CS10 cells. Growth and serum PSA level of LNCaP-CS10 tumors were not inhibited by 10 or 100 mg/kg bicalutamide, whereas tumor growth and the serum PSA level in LNCaP xenografts were suppressed by 10 or 100 mg/kg bicalutamide (Fig. 5). I consider the reason why the LNCaP-CS10 tumor growth-inducing (agonist) activity of bicalutamide detected in the *in vitro* assay was not detected in this xenograft model to be the low level of adrenal gland-derived androgen remaining in the castrated mice. From these lines of evidence, LNCaP-CS10 was designated as an “outlaw” castration-resistant prostate cancer cell line.

To investigate the mechanism of the resistance of LNCaP-CS10, I analyzed the effects of bicalutamide on growth and PSA production. To my surprise, bicalutamide induced the growth and PSA production of LNCaP-CS10 (Fig. 2C and D). The results

indicate that bicalutamide acted as an agonist in LNCaP-CS10 cells but not in LNCaP cells. Induction of nuclear AR by bicalutamide was more notable in LNCaP-CS10 cells than in LNCaP cells (Fig. 2E), although the total AR protein level was not increased in the cells. Bicalutamide is reported to translocate the transcriptionally inactive AR to the nuclei of LNCaP cells (14,33). But, the fact that bicalutamide acted as an agonist on growth and PSA production in LNCaP-CS10 suggests that the induction activity of bicalutamide on AR nuclear translocation contributed to the AR agonist activity of bicalutamide in LNCaP-CS10.

Various mechanisms of castration resistance in outlaw tumors have been suggested such as activation of AR by deregulated cytokines and growth factors, including IL-6, IGF-1, EGF, KGF, through the JAK-STAT, Akt, MAPK pathways in the absence of androgen (27,34-38). To investigate the mechanism of outlaw activation in LNCaP-CS10, I first analyzed AKT and ERK status but found no apparent difference between LNCaP-CS10 and LNCaP for pAKT or pERK (data not shown). Next, the comparative genomic hybridization analysis did not reveal any of the copy number changes in the PTEN gene known to be the AKT pathway regulator and frequently deleted in prostate cancer cells (39,40). In addition, gene expression profiling and western blotting showed an overexpression of the IL-6Rα gene and protein in LNCaP-CS10. But the cell proliferation assay revealed that the anti-IL-6 neutralizing antibody and anti-IL-6Rα neutralizing antibody did not affect the growth of LNCaP-CS10 in the absence of androgen, indicating that IL-6 axis activation is not a mechanism of the outlaw activation of LNCaP-CS10 (data not shown).

Lastly, I investigated the downstream of AR signaling in LNCaP-CS10 cells. The mitotic signaling of the AR is thought to ultimately target the cell cycle machinery (41). Androgen stimulates the expression of the cell cycle genes, cyclin-dependent kinase 1 (CDK-1), CDK-2 and CDK-4, and increases the levels of cyclin A and cyclin B1 mRNAs (42,43). Therefore, I analyzed the expression of androgen-regulated CDKs and cyclins using gene chip analysis under an androgen-depleted condition but found no apparent differences between LNCaP-CS10 and LNCaP for CDK-1, CDK-2, CDK-4, cyclin A2 and cyclin B1 (data not shown). Further study is necessary to elucidate the exact mechanism of the resistance of LNCaP-CS10.

LNCaP-CS10 showed characteristics different from other bicalutamide-resistant cell lines previously reported. LNCaP-abl, which was derived from LNCaP and established after long-term culture in an androgen-depleted medium, showed AR overexpression and did not have a newly altered AR sequence (30). And LNCaP-abl cells showed hypersensitivity to androgen and induction by bicalutamide on cell growth.

LNCaP-CDXRs established in an androgen-depleted medium with 5 μM bicalutamide showed AR overexpression (31). LNCaP-CDXR cell growth was unaffected by bicalutamide but was repressed by androgen. LNCaP-BICs, established in an androgen-depleted medium with 10 pM R1881 and 1 μM bicalutamide for 3 months, did not show AR overexpression or alteration of the AR sequence, and its cell growth was unaffected by bicalutamide or R1881. Thus LNCaP-BICs were designated as AR by-pass models (44). On the other hand, LNCaP-CS10 did not show AR overexpression but cell growth was induced by both bicalutamide and R1881. KUCaP was established by transplanting liver metastatic tissue into male SCID mice (22). Tissue samples were obtained from a patient who was treated with total androgen blockade using bicalutamide. This cell line has W741C mutation in the ligand-binding domain of AR. LNCaP-cxDs established in an androgen-depleted medium with 1 μM or 0.1 μM bicalutamide have W741C or W741L mutation of AR in addition to the T877A mutation (32). In KUCaP and LNCaP-cxDs models, bicalutamide acts as an agonist as a result of the mutations. On the other hand, LNCaP-CS10 does not have these new mutations.

In conclusion, I established a bicalutamide-resistant cell line, LNCaP-CS10, with outlaw pathway activation and is applicable to xenograft experiments. LNCaP-CS10 is useful for the elucidation of the outlaw pathway mechanisms of castration resistance and the evaluation of the efficacy of new therapeutics for castration-resistant prostate cancer.

Chapter II

Significance of AR nuclear translocation in castration-resistant prostate cancer cells

Introduction

AR is a transcription factor that is known to be ligand-specifically activated. Unliganded AR, primarily located in the cytoplasm, translocates into the nucleus following androgen binding where it binds to specific DNA sequences and subsequently activates the transcription of its target genes (14). Expression of AR and prostate-specific antigen (PSA) persists in most CRPC specimens (8-10), suggesting that the AR signal remains obstinately active. Therefore, AR is still considered to be a potential therapeutic target for CRPC. Pienta *et al*. (13) proposed three major AR-related mechanisms of castration resistance (Fig. 1): i) hypersensitivity to androgen due to overexpression of AR (15-17); ii) loss of ligand specificity following an AR mutation (18-22); and iii) androgen-independent activation of AR mediated by deregulated growth factors and cytokines, termed "outlaw" activation (23-29). My collaborators and I have previously reported that existing AR antagonists, such as bicalutamide, have major limitations in that they have a so-called partial agonist profile (45,46). Specifically, those drugs have agonistic activity in addition to the intended antagonistic activity. Because of that, the drugs show no or at most weak antitumor effect against prostate cancers with castration resistance acquired by the three abovementioned mechanisms (32,46).

It is well known that tamoxifen, an ERα antagonist widely used against breast cancers, also has a partial agonist profile and tumors can develop resistance to tamoxifen (47). To conquer the problem, ERα pure antagonists, such as fulvestrant (Faslodex), have been introduced and demonstrated to be efficacious against tamoxifen-resistant breast cancers (48-50). In an analogous fashion to ERα, I hypothesized that an AR antagonist without agonistic activity, namely an AR pure antagonist, would be efficacious against CRPC.

Tran *et al*. (51) reported a new compound effective against CRPC with AR-overexpression. The compound reduced the efficiency of AR nuclear translocation. However, the compound was reported to have a partial agonist activity and could not completely inhibit the translocation of AR into the nucleus. In light of those findings, and taking into account the successful experience with ERα pure antagonists, my coworkers and I screened approximately 2000 compounds for their properties as an AR nuclear translocation pure antagonist, and discovered a new compound, CH5137291, showing high-quality properties (52,53).

In the present study, I minutely examined the characteristics of CH5137291 as well as its antitumor activity in comparison with that of bicalutamide in several CRPC models with different mechanisms of resistance. In fact, I investigated the significance of AR nuclear translocation in CRPC which has the three major mechanisms, overexpression of AR, mutations in AR genes, and androgen-independent activation of AR.

Materials and Methods

Reagents

CH5137291 and fulvestrant were synthesized in my laboratories. Bicalutamide was purchased as Casodex tablets (AstraZeneca, London, UK) and purified in my laboratories. Synthetic androgen R1881 was purchased from NEN Life Science Products (Boston, MA, USA). Progesterone, mifepristone, dexamethasone, and β-estradiol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aldosterone was purchased from Acros Organics (Geel, Belgium). Abiraterone acetate was synthesized in my laboratories.

Cells

LNCaP, VCaP, PC3, HeLa, and COS-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). LNCaP-BC2 and LNCaP-CS10 cells were previously established in my laboratory (46). LuCaP35V cells that progressed in castrated male mice and expressed wild-type AR were a gift from Prof. Robert L Vessella (University of Washington, Seattle, WA, USA) (54). LNCaP cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. VCaP cells were maintained in DMEM (high glucose) (Invitrogen) supplemented with 10% FBS. LNCaP-BC2 cells were maintained in phenol red-free RPMI-1640 supplemented with 10% FBS and 2 μM bicalutamide. LNCaP-CS10 cells were maintained in phenol red-free RPMI-1640 supplemented with 10% dextran-coated charcoal-treated FBS (DCC-FBS; Hyclone Laboratories, Logan, UT, USA) and 10 μM bicalutamide. PC3 cells were maintained in F-12 Kaighn's medium (Invitrogen) supplemented with 10% FBS. HeLa cells were maintained in EMEM medium (Sigma-Aldrich) supplemented with 10% FBS, 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and 1.5 g/L sodium bicarbonate (Sigma-Aldrich). COS-7 cells were maintained in DMEM (high glucose) supplemented with 10% FBS, 1.5 mM L-glutamine (Sigma-Aldrich), 4.5 g/L D-glucose (Sigma-Aldrich), and 1.5 g/L sodium bicarbonate.

The characteristics in terms of hormone sensitivity, AR status, and AR mutation of these cell lines are summarized in Table I

Animals

Five-week-old male C.B-17/Icr-*scid* Jcl severe combined immune-deficient (SCID) mice were purchased from CLEA Japan (Tokyo, Japan). Four- to 6-year-old cynomolgus monkeys (*Macaca fascicularis*) were bred in the Chugai Research Institute for Medical Science (Tokyo, Japan). All animals were housed in a pathogen-free environment under controlled conditions (temperature 20–26°C, humidity 35–75%, light/dark cycle 12/12 h). Chlorinated water and irradiated food were provided *ad libitum*. The health of the animals was monitored by daily observation. The protocols of the animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd., and all animal experiments were performed in accordance with the Guidelines for the Accommodation and Care of Laboratory Animals promulgated in Chugai Pharmaceutical Co., Ltd.

Fluorescence imaging analysis

LNCaP cells in 10% DCC-FBS medium were plated 24 h before transfection. To analyze intracellular translocation of AR, I used HaloTag system. The cells were transfected with HaloTag pHT2-hAR-WT, HaloTag pHT2-W741C, or HaloTag pHT2-T877A using Fugene HD transfection reagent (Roche, Basel, Switzerland). Forty-eight hours after the transfection, HaloTag TMR ligand (Promega, Fitchburg, WI, USA) was added, and the cells were incubated for 0.5 h. CH5137291 or bicalutamide (10 μM) was added in the presence or absence of R1881 (0.5 nM). After 2 h incubation, fluorescence imaging of the living cells was performed with a BioZero fluorescence microscope (Keyence, Osaka, Japan).

Protein fractionation and western blotting

Cells were plated onto poly-D-lysine coated plates (Becton Dickinson, Heidelberg, Germany) in 10% DCC-FBS medium. After 3-day incubation, 10 μM of CH5137291 or bicalutamide was added and the plates were incubated for another 24 h. Then, nuclear and cytoplasmic fractions were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA), and a total fraction was prepared using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche). AR-specific antibody N-20, IkBα-specific antibody C-15, and c-Jun-specific antibody H-79 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunoblotting.

Cell proliferation assay

VCaP, LNCaP, LNCaP-BC2, and LNCaP-CS10 cells were pre-incubated in phenol red-free RPMI-1640 medium supplemented with 10% DCC-FBS for 2 to 4 days and were then plated onto poly-D-lysine-coated plates (Becton Dickinson) (VCaP, 1×10^4 cells/well; LNCaP, LNCaP-BC2, and LNCaP-CS10, 5×10^3 cells/well) in 10% DCC-FBS medium. After overnight incubation, CH5137291 or bicalutamide at concentrations of 4.6 nM to 30 μM was added in the presence of R1881 (VCaP, 0.1 nM; LNCaP, 0.1 nM; LNCaP-BC2, 0.01 nM) or absence of R1881 (LNCaP-CS10). After an additional 10 days' incubation, the number of remaining cells were estimated using a DNA quantity assay (FluoReporter Blue Fluorometric dsDNA Quantitation Kit; Invitrogen).

Reporter gene assay of AR

The following plasmids were used: GMLUC, an MMTV–luciferase reporter plasmid created by replacing the reporter of GMCAT (ATCC) with luciferase; phRL-CMV (Promega), a *Renilla* luciferase plasmid; and human AR expression plasmids corresponding to mutations (pcDNA3.1-hAR-WT, pcDNA3.1-hAR-W741C, and pcDNA3.1-hAR-T877A).

Twenty-four hours before transfection, the PC3 cells in 10% DCC-FCS medium were plated onto 96-well plates at 1×10^4 cells/well. The cells were co-transfected with GMLUC (50 ng/well), phRL-CMV (0.5 ng/well), and human AR expression plasmid (10 ng/well) using a TransIT-Prostate Transfection Kit (Mirus, Madison, WI, USA). Six hours after the transfection, various doses of CH5137291 or bicalutamide were added in the presence of R1881 (WT, 0.05 nM; W741C, 0.5 nM; T877A, 0.5 nM) or absence of R1881. Cell lysates were collected 48 h after the treatment with the compounds, and the luciferase activity of each sample was measured using a Dual-Luciferase Reporter Assay System (Promega).

Reporter gene assay of PR, GR, MR, and ERα

Twenty-four hours before transfection, HeLa cells in 10% DCC-FBS medium were plated onto 96-well plates at 1×10^4 cells/well for the assays of PR and ERα. COS-7 cells were plated at 0.75×10^3 cells/well for the assays of GR and MR. The cells were co-transfected with GMLUC (50 ng/well), phRL-CMV (0.5 ng/well), and expression plasmids of the nuclear receptors (10 ng/well of pSG5-hPR, pRShGR, or pRShMR) using Fugene 6 transfection reagent (Roche). For ERα, the cells were co-transfected with ERE-reporter vector (50 ng/well), phRL-CMV (0.5 ng/well), and ERα expression

vector HEG0 (1 ng/well) using Fugene 6 transfection reagent. Six hours after transfection, CH5137291 or bicalutamide (10 μM) were added in the presence or absence of the corresponding agonist (10 nM progesterone for PR, 10 nM dexamethasone for GR, 1 nM aldosterone for MR, and 1 nM estradiol for ERα). Cell lysates were collected 48 h after treatment with the compounds. Mifepristone (1 nM), mifepristone (10 nM), progesterone (10 nM), and fulvestrant (100 nM) were used as positive antagonists for PR, GR, MR, and ERα, respectively.

Docking models analysis

Models of CH5137291 docking with wild-type and W741C-mutant ARs were built based on the X-ray crystal structure of the W741L-mutant AR in complex with bicalutamide (Protein Data Bank accession code:1z95). The three-dimensional structure of CH5137291 was modeled using Sybyl software (Tripos Inc., St.Louis, USA) with a Tripos force field.

In the docking model of CH5137291 for W741C-mutant AR, CH5137291 was manually docked to the W741C-mutant AR such that (i) the crystallographic structure of the W741C-mutant AR was fixed, (ii) helix 12 of the AR was removed from the AR model because of the intensive collision between CH5137291 and helix 12, (iii) the crystallographic structure of the trifluoromethyl benzonitrile moiety of bicalutamide and its binding mode were used for CH5137291 and kept fixed during the docking, (iv) the binding mode of the portion other than the trifluoromethyl benzonitrile moiety of CH5137291 was manually determined so as to avoid a steric collision between CH5137291 and the AR without helix 12, and (v) energy minimization of the "compound/AR without helix 12" complex was performed using a molecular mechanics method with the Tripos force field, on condition that the coordinates of AR without helix 12 were fixed. After the energy minimization, the crystallographic coordinates of helix 12 were re-built into the AR structure.

The docking models of CH5137291 or bicalutamide for wild-type AR were built based on the docking model of CH5137291 or bicalutamide for W741C-mutant AR, respectively. C741 was changed to W741 such that the conformation of W741 of the model would be identical to that of the crystallographic structure of W741 of wild-type AR (PDB ID: 1e3g).

Efficacy experiments in mouse xenograft models

Xenograft models were prepared and plasma PSA was quantified as described previously (46). Briefly, LNCaP (2×10^6 cells), LNCaP-BC2 (2×10^6 cells),

LNCaP-CS10 (2×10^6 cells), or LuCaP35V (blocks of xenografted tumor) was subcutaneously inoculated into non-castrated or castrated 6- to 8-week-old male SCID mice. When the tumor size reached 90–400 mm^3, the animals were randomized into control and treatment groups. The agents or vehicle (5% gum arabic; Sigma-Aldrich) were orally administered at 10 or 100 mg/kg once a day for 2–17 cycles of 5 days on/2 days off starting from the day of randomization. The antitumor efficacy was evaluated by tumor volume (TV) and the percentage of tumor growth inhibition (TGI%). TV was estimated by using the equation $V = ab^2/2$, where a and b are the length and width of the tumor, respectively. TGI% was calculated as follows: TGI% = [1 − (mean change in TV in each group treated with antitumor drugs/mean change in TV in control group)] × 100. The plasma PSA levels were measured by ELISA (Eiken Chemical, Tokyo, Japan). Relative PSA concentration (%) = (PSA concentration/PSA concentration on Day 0 of administration) × 100. Serum testosterone concentrations were measured using LC/MS/MS (ASKA Pharma Medical, Tokyo, Japan) at the end of the experiment.

To evaluate the efficacy of CH5137291 on tumors that had become resistant to bicalutamide, LNCaP-xenografted non-castrated mice were orally administered bicalutamide (100 mg/kg) until resistant tumors appeared. The mice bearing resistant tumors were selected, re-randomized into two groups, and moved on to secondary treatment. Bicalutamide (100 mg/kg) was administered in one group and CH5137291 (100 mg/kg) in the other group. The tumors with acquired resistance were defined as those that fulfilled both of the following two criteria: (1) (PSA concentration on Day 10 of administration/PSA concentration on Day 0 of administration) × 100 < 85%, and (2) the tumor volume on the day of re-randomization/minimum tumor volume during primary treatment > 1.4.

Exposure and serum PSA in cynomolgus monkeys

For the exposure assay, the monkeys received a single oral administration of CH5137291 at doses of 0.1, 1, 10, or 100 mg/kg. The vehicle was 1% hydroxypropylcellulose (Nippon Soda, Tokyo, Japan). The serum concentration of CH5137291 was measured by using LC/MS/MS (API3200; Applied Biosystems, Foster City, CA, USA). To assess the effect of CH5137291 on serum PSA, I selected 4- to 6-year-old monkeys with initial serum PSA concentrations of >0.25 ng/mL, as measured by using chemiluminescent immunoassay at BML, Inc. (Tokyo, Japan). The animals were randomized, and CH5137291 was orally administered daily for 7 days at doses of 0.1, 1, 10, or 100 mg/kg. Twenty-four hours after the last administration, serum was harvested and PSA concentration was measured.

Results

Inhibition of AR nuclear translocation by CH5137291

Kawata *et al*. (53) reported the inhibitory effect of CH5137291 on AR nuclear translocation by using COS-7 cells transiently transfected with human wild-type AR. In the present study, I examined the effect of CH5137291 on subcellular localization in prostate cancer cell lines with wild-type or mutated AR. For this purpose, I used LNCaP cells transfected with HaloTag-fused wild-type AR, W741C-mutant AR, or T877A-mutant AR. In each of these cells with three different types of AR, CH5137291 clearly inhibited AR nuclear translocation in the presence of synthetic androgen R1881. In addition, CH5137291 in the absence of R1881 did not induce AR nuclear translocation suggesting that CH5137291 does not have an agonistic effect on AR (Fig. 6A). In contrast, bicalutamide did not inhibit AR nuclear translocation in the presence of R1881 and induced AR nuclear translocation in the absence of R1881.

Next, I examined the effect of CH5137291, in comparison with bicalutamide, on the intracellular distribution of AR protein. For this purpose, LNCaP-CS10 cells were used, because AR in LNCaP-CS10 cells is androgen-independently activated. Bicalutamide reportedly acts as a full agonist on LNCaP-CS10 cells. I found that CH5137291 decreased the nuclear AR level and increased the cytoplasmic AR level without affecting the total AR level (Fig. 6B). On the other hand, as was expected, bicalutamide increased the nuclear AR level but did not affect the total AR level.

These results indicate that CH5137291 acts as an AR nuclear translocation inhibitor, not only in androgen-dependent prostate cancer cells with wild-type or mutant AR but also in androgen-independent prostate cancer cells having outlaw pathways.

Cell growth inhibition by CH5137291 against hormone-sensitive prostate cancers and CRPCs in vitro

The proliferation inhibitory activity of CH5137291, compared with bicalutamide, was examined *in vitro* using four prostate cancer cell lines (VCaP, LNCaP, LNCaP-BC2, and LNCaP-CS10) each with different hormone sensitivity and AR status (Table I, Fig. 7). The assays were all performed in the presence of optimal concentrations of synthetic androgen R1881 except for LNCaP-CS10, because LNCaP-CS10 does not require androgen for its proliferation.

In VCaP cells, growth was completely inhibited by CH5137291 at concentrations of 3 to 30 μM. In contrast, bicalutamide almost completely inhibited the cell growth at

3 μM, however at 30 μM, it induced cell growth. In LNCaP cells, CH5137291 inhibited cell growth more strongly than bicalutamide, and completely inhibited cell growth at concentrations of 1–30 μM. In LNCaP-BC2 cells, CH5137291 also showed stronger inhibition of cell growth than bicalutamide, and complete inhibition was observed at CH5137291 concentrations as low as 0.3 μM. Although bicalutamide showed strong inhibition of cell growth at 3 μM, cell growth was revived at higher concentrations. In LNCaP-CS10 cells, CH5137291 showed strong inhibition of cell growth, whereas bicalutamide enhanced cell growth.

Antitumor activity of CH5137291 against CRPC xenografts

The *in vivo* efficacy of CH5137291 on CRPCs was examined by using xenograft models of castrated SCID mice bearing LNCaP-BC2 and LNCaP-CS10. CH5137291 treatment (10 and 100 mg/kg) potently inhibited tumor growth in both xenograft models. The TGI% in each model was as follows: LNCaP-BC2, 104% and 104% on Day 17 (at 10 and 100 mg/kg CH5137291, respectively); LNCaP-CS10, 88% and 88% on Day 16 (at 10 and 100 mg/kg CH5137291, respectively) (Fig. 8). The plasma PSA level was also measured as a pharmacodynamic biomarker of AR antagonist activity. In both of the xenograft models tested, CH5137291 completely inhibited the increase in plasma PSA level, even at the dose of 10 mg/kg, without agonistic activity (Fig. 8). In contrast, bicalutamide treatment (10 and 100 mg/kg) showed almost no or little effect on either TV or PSA level even at the dose of 100 mg/kg.

Pure antagonistic effect of CH5137291 on the transcriptional activity of wild-type and mutant AR

The antagonistic effect of CH5137291 on AR transcriptional activity was examined by reporter gene assay. For this purpose, PC3 cells transiently co-transfected with AR (wild-type, W741C, or T877A) expression vectors and GMLUC reporter vectors including an MMTV promoter, a well-characterized AR-targeting promoter, were used. The W741C and T877A mutations are known to cause resistance to bicalutamide and flutamide, respectively (19,22,32).

In all of the three transfectants, CH5137291 inhibited transcriptional activity of AR in a dose-dependent manner in the presence of R1881 (Figs. 9A, B, C). Bicalutamide showed different behaviors in the three transfectants. With wild-type AR, bicalutamide showed dose-dependent inhibition in transcriptional activity up to 3 μM; however, resurgence was observed at higher bicalutamide concentrations (Fig. 9A). With W741C-mutant AR, bicalutamide had no inhibitory effect throughout the concentrations

used (Fig. 9B). With T877A-mutant AR, bicalutamide showed a weaker inhibitory effect compared with CH5137291. In the absence of R1881, CH5137291 showed no agonistic effect on the transcriptional activity of AR in any of the three transfectants (Figs. 9A, B, C). In contrast, bicalutamide strongly increased the transcriptional activity of W741C-mutant AR in a dose-dependent manner (Fig. 9B). With wild-type and T877A-mutant AR, bicalutamide increased the transcriptional activity at relatively high concentrations (Figs. 9A, C).

Complete inhibition of helix 12 folding by CH5137291 in wild-type and W741C-mutant AR

The folding of helix 12, present in the ligand-binding domain of AR, is reported to be necessary for a ligand-induced agonist effect on AR transcriptional activity (11,12). The crystal structure of the bicalutamide/W741C-mutant AR complex revealed that bicalutamide does not collide with the C741 of the AR as it does with W741 in the wild-type AR; therefore, helix 12 of the bicalutamide/W741C-mutant AR complex is folded in the same manner as when a ligand, such as R1881, binds to it (Fig. 10) (55). Reportedly, this is a reason for bicalutamide exhibiting an agonist effect in W741C-mutant AR. To investigate the differences in the mechanisms of CH5137291 and bicalutamide with respect to agonist/antagonist effects against wild-type or W741C-mutant ARs, I examined a docking model of CH5137291 against wild-type and W741C-mutant ARs. The docking model indicated that the terminal sulfonamide group of CH5137291 intensively collided with the M895 residue of helix 12 in both the wild-type AR and W741C-mutant AR. The collision would cause a complete inhibition of helix 12 folding and would result in a pure antagonist effect in both wild-type and W741C-mutant ARs. The varying interactions between the compound and AR may lead to the differing agonist and antagonist characteristics of CH5137291 and bicalutamide, respectively, in wild-type and W741C-mutant ARs.

Nuclear receptor specificity of CH5137291

Because of the highly similar structures within the nuclear receptor superfamily, I used a reporter gene assay to profile the effect of CH5137291 on the transcriptional activity of other nuclear receptors, including PR, GR, MR, and ERα, in the presence or absence of ligands. CH5137291 exhibited weaker antagonistic effects on the PR as compared to bicalutamide. Moreover, it exhibited neither agonistic nor antagonistic effects on GR, MR, or ERα (Fig. 11).

Antitumor activity of CH5137291 in LuCaP35V xenografts that progressed after castration and in LNCaP xenografts that progressed during bicalutamide treatment

The effects of CH5137291 was examined against LuCaP35V xenografted tumors that progressed in castrated mice and LNCaP xenografted tumors that had failed to respond to initial bicalutamide treatment. In the LuCaP35V xenograft model, CH5137291 treatment (10 and 100 mg/kg) potently inhibited the tumor growth and plasma PSA level. The TGI% was 82% and 85% on Day 48 (at 10 and 100 mg/kg, respectively) (Fig. 12A). In contrast, bicalutamide treatment (10 and 100 mg/kg) showed almost no effect on TV or PSA level. In the LNCaP xenograft model, 50 days after initiation of bicalutamide treatment, animals with tumors that showed resistance to bicalutamide were selected and randomized into two groups. One group continued receiving bicalutamide and the other group was switched to CH5137291 treatment. Only in the group switched to CH5137291 treatment did the tumor growth become static and the plasma PSA level decrease (Fig. 12B).

Long-term tumor growth inhibition by CH5137291 in the hormone-sensitive LNCaP xenograft model

Long-term tumor growth inhibition against hormone-sensitive prostate cancer was examined by using an LNCaP xenograft in non-castrated SCID mice (Fig. 12C). The average time taken for the tumor volume to exceed double the initial volume was 113 days in the CH5137291 group and 58 days in the bicalutamide group. In addition, CH5137291 suppressed the plasma PSA concentration and the reduction in body weight during the observation period. These results indicate that CH5137291 can control tumor growth of hormone-sensitive prostate cancer for nearly twice as long as the period of control with bicalutamide.

Exposure of CH5137291 and serum PSA levels in cynomolgus monkeys

Finally, to estimate the clinical therapeutic potential of CH5137291, cynomolgus monkeys were treated with CH5137291. Serum concentration of the drug and PSA as a pharmacodynamic biomarker was measured. The CH5137291 concentration dose-dependently increased. PSA concentration decreased in inverse proportion to the dosage of CH5137291 with a maximum 80% inhibition at 100 mg/kg (Fig. 13).

Discussion

Three major AR-related mechanisms of castration resistance in prostate cancer have been reported: i) hypersensitivity to androgen caused by AR overexpression, ii) loss of ligand specificity due to AR mutation; and iii) androgen-independent activation of AR mediated by deregulated growth factors and cytokines (Fig. 1) (13).

Following binding with androgen, AR exhibits transcriptional activity through dimerization, nuclear translocation, and binding to the androgen response element. Each of these processes is essential for AR signaling. Among these processes, My coworkers and I focused on the nuclear translocation, and screened for compounds that inhibited nuclear translocation of AR without agonistic activity, resulting in the finding of.a candidate: CH5137291. In the present study, I conducted a detailed investigation into the inhibitory activity of CH5137291 on nuclear translocation of AR. I found that CH5137291 inhibited—regardless of the presence of androgen—the nuclear translocation of wild-type, bicalutamide-resistant type (W741C), and flutamide-resistant type (T877A) ARs which were exogenously expressed in LNCaP cells. In contrast, bicalutamide induced the nuclear translocation of all of the above AR types, similar to the action of androgen (Fig. 6A). I also confirmed that CH5137291 inhibited the nuclear translocation of the AR that was endogenously expressed in LNCaP-CS10 (Fig. 6B).

Because the nuclear translocation of AR is required for AR signaling in prostate cancer, it was considered that the inhibition of AR nuclear translocation was the most important characteristic of CH5137291.

To investigate the importance of nuclear translocation of AR in the three mechanisms of castration resistance, I used CH5137291, an AR nuclear translocation inhibiting compound, and the following cells corresponding to each mechanism of resistance: i) LNCaP-BC2 cells as an AR overexpression model, ii) LNCaP-CS10 cells as an androgen-independent AR activation model, and iii) PC3 cells expressing mutant ARs for reporter gene assay as an AR mutation model. LNCaP-BC2 cells can proliferate at androgen concentrations as low as 1/10 of those required for parental LNCaP cells *in vitro*, and can grow in castrated male mice (46). LNCaP-CS10 cells proliferate AR-dependently in the absence of androgen *in vitro*, and can grow in castrated male mice.

In *in vitro* studies, CH5137291 completely inhibited the cell growth of both LNCaP-BC2 and LNCaP-CS10 cells; however, bicalutamide showed a biphasic activity on the proliferation of LNCaP-BC2 cells, and surprisingly, it stimulated the

proliferation of LNCaP-CS10 cells even at the lower concentrations tested *in vitro* (Fig. 7B). In xenograft models of castrated mice with these cell lines, CH5137291 showed significant antitumor activity along with a reduction in plasma PSA levels (Figs. 8A, B). In contrast, bicalutamide did not inhibit tumor growth at all in these xenograft models. These results coincide well with our previous reports (46), and suggest that CH5137291 will be effective on those castration resistant prostate cancers which have AR overexpression or androgen-independent activation of AR and do not respond to bicalutamide.

The reporter gene assays performed using different types of mutant AR clearly showed that bicalutamide acts as a full agonist on the transcriptional activity in bicalutamide-resistant W741C-mutant AR, and as a partial agonist in wild-type AR and flutamide-resistant T877A-mutant AR (Figs. 9A, B, C) (19,22,32). In contrast, CH5137291 acted as a pure antagonist on the transcriptional activity in each of the ARs (Figs. 9A, B, C). These results suggest that CH5137291 is potentially superior to bicalutamide as an AR antagonist against all of the three mechanisms of castration resistance.

Because the inhibition of AR nuclear translocation by CH5137291 induced the strong antitumor activity or anti AR transcriptional activity in CRPCs, it was considered that the nuclear translocation of AR is required for all three of the abovementioned mechanisms of castration resistance.

To elucidate the pure AR antagonistic nature of CH5137291 in terms of three-dimensional structure, a docking model analysis was performed. The folding of helix 12 of AR is reported to be caused by ligand binding and is considered necessary for a ligand-induced AR agonist effect (11,12). The docking model revealed that CH5137291 intensively collided with the M895 residue of helix 12 in both wild-type AR and W741C-mutant AR (Fig. 10). This collision of CH5137291 would cause a complete inhibition of helix 12 folding and would result in a pure antagonist effect in both the wild-type AR and W741C-mutant AR. On the other hand, bicalutamide collided with the W741 residue in the wild-type AR, but did not collide with the C741 residue in the W741C-mutant AR; subsequently, helix 12 of the bicalutamide/W741C-mutant AR complex was folded in the same manner as when the ligand binds to wild-type AR (55). This would be one of the reasons for the partial agonistic effect of bicalutamide in wild-type AR and its fully agonistic effect in W741C-mutant AR.

I would like to take other step to discuss the AR nuclear translocation inhibiting compound as the therapeutic agent for CRPC. Recently, reports regarding the

development of the CYP17 inhibitor abiraterone acetate and the AR antagonist MDV3100 have described their efficacy against CRPC (51,56-58). My LNCaP-CS10 xenograft study showed that abiraterone acetate did not exert an effect on CRPC with an androgen-independent AR activation mechanism of resistance because the mode of action of the agent is the inhibition of androgen synthesis, whereas LNCaP-CS10 tumors grow in an androgen-independent manner (data not shown). MDV3100 is reported to have partial agonist activity on AR nuclear translocation (51); on the other hand, CH5137291 exhibited pure antagonist activity on AR nuclear translocation. These data suggest the advantages of CH5137291 over other agents against CRPCs.

In addition to superior non-clinical efficacy, fewer adverse effects and higher exposure in humans are indispensable for the development of a compound for therapeutic use. Because AR belongs to the nuclear steroid receptor family and has a structure highly similar to that of other nuclear receptors (e.g., PR, GR, MR, and ERs), any drug targeting AR holds the possibility for cross reactivity-related adverse effects (59,60). My results indicated that CH5137291 exhibited weaker antagonistic effects on the PR as compared to bicalutamide and did not exhibit any agonist/antagonist effects on GR, MR, or ERα (Fig. 11A, B, C, D). I therefore consider that CH5137291 would not cause any adverse events through cross reactivity with other nuclear receptors.

I also examined the effectiveness of CH5137291 in various therapeutic situations. To mimic second-line hormonal therapy, I used a castration-resistant LuCaP35V and LNCaP xenograft models (54). In the latter model, treatment was changed to CH5137291 when the tumor became resistant to bicalutamide after initial tumor growth inhibition by bicalutamide. CH5137291 showed antitumor activity against both of the two models (Fig. 12A, B). To mimic first-line hormonal therapy, the duration of tumor stabilization in the LNCaP xenograft model was examined. CH5137291 showed long-term antitumor efficacy as compared to bicalutamide (Fig. 12C). These results suggest that CH5137291 was potentially efficacious not only against CRPCs that progress despite castration or bicalutamide treatment but also against hormone therapy-naïve prostate cancers.

Concerning exposure, other pure AR antagonists such as CH4892280 have failed to achieve adequate plasma levels due to their metabolic instability (11,45). However, based on the present results, sufficient exposure of CH5137291 is expected in humans by virtue of its remarkable stability in the liver microsomes of mice, rats, monkeys, and humans ((53) and data not shown) as well as the dose-dependent increase in levels in plasma/serum, which were adequately efficacious not only in mice xenografts but also

in cynomolgus monkeys (Fig. 13). These results suggest that CH5137291 shows promising clinical efficacy.

General discussion

Three mechanisms of castration resistance have been suggested to date: i) overexpression of AR, ii) mutation of AR, and iii) androgen-independent AR activation (13). Since several cell line models have already been established for investigating mechanisms i) and ii) (17,22,30-32), they have been widely proven with molecular and cellular biological analyses (15-22). However, mechanism iii) has not been extensively analyzed in a molecular/cellular biological framework because of the lack of an appropriate cell line model, and thus has not yet been clearly proven.

Nuclear translocation of AR is considered to be a common signaling pathway in HSPC as well as in these three CRPCs. Therefore, I hypothesized and investigated that inhibition of AR nuclear translocation would inhibit the proliferation of all three types of CRPC, and investigated the significance of AR nuclear translocation in CRPCs with the above three major mechanisms.

Prior to starting the investigation, I tried to establish a CRPC cell line with the mechanism of androgen-independent AR activation, which had not been experimentally explored in detail previously. The LNCaP-CS10 cell line was established by culturing the HSPC cell line LNCaP in a medium supplemented with 10% FBS from which the steroid hormone was removed and 10 μM of bicalutamide for 4 months. In LNCaP-CS10, cell proliferation and PSA expression increased in the absence of androgen, although AR overexpression and additional mutation were not observed. In the *in vivo* LNCaP-CS10 xenograft model, the same growth rate was observed under non-castration conditions and castration conditions, and inhibition of tumor growth and PSA expression was not observed when bicalutamide or abiraterone, testosterone synthesis inhibitor, was administered. In addition, LNCaP-CS10 showed suppressed proliferation and PSA expression when AR was knocked down by siRNA. These data indicated that LNCaP-CS10 was a CRPC cell line with a mechanism of androgen-independent AR activation. Since the mechanism of androgen-independent AR nuclear translocation in LNCaP-CS10 cells has not been elucidated, further detailed analysis is required.

Next, to investigate the influence of blocking AR activity through inhibition of AR nuclear translocation in CRPC, my collaborators and I developed the compound CH5137291, which inhibits the AR nuclear translocation. CH5137291 was screened from 2000 compounds based on the inhibitory effects of wild-type and mutant AR transcription activity, AR nuclear translocation, and proliferation of CRPC cells, and

observation of a conformational change of AR through modeling as indicators (52,53). In this study, I used the LNCaP-BC2 cell line as an AR-overexpression model, the PC3 cell line in which W741C or T877A mutant AR was transiently transfected as an AR mutation model, and the LNCaP-CS10 cell line as an androgen-independent AR activation model (46).

In LNCaP-CS10 cells, CH5137291 more strongly suppressed the nuclear translocation of endogenous AR, cell proliferation *in vitro*, tumor growth, and PSA expression *in vivo* compared to bicalutamide. Furthermore, CH5137291 strongly inhibited the nuclear translocation and transcriptional activity of W741C or T877A mutant AR, which causes castration resistance, and of wild-type AR. Androgen-dependent AR transcriptional activation is caused by the helix 12 folding of AR (11,12). According to the docking model analysis, CH5137291 is suggested to compete with androgen for binding, which inhibits the helix 12 folding of AR by immediate interference with the M895 residue both in the wild-type AR and in the W741C mutant AR. In addition, CH5137291 inhibited tumor growth and PSA expression *in vivo* against the AR-overexpressing LNCaP-BC2 cell line. Based on these findings, it was shown that tumor proliferation is strongly suppressed when AR transcriptional activity is completely blocked by inhibition of AR nuclear translocation in CRPC models with the above-mentioned three mechanisms.

In addition to its superior non-clinical efficacy, CH5137291 was suggested to have no adverse effects, because it exhibited weaker antagonistic effects on PR as compared to bicalutamide and did not exhibit any agonist/antagonist effects on GR, MR, or ERα. The lack of side effects associated with cross-reactivity to these nuclear receptors is an important feature of CH5137291 in therapeutic applications (59,60).

Finally, to predict the effect of the AR nuclear translocation inhibitor CH5137291 in clinical prostate cancer, the following tests were conducted. First, to investigate its potential in secondary therapy, I investigated the effect of CH5137291 on tumor re-growth after administration of bicalutamide using the *in vivo* LNCaP xenograft model. As a result, tumor regrowth was strongly suppressed. Next, to examine its potential in primary therapy, the effect of CH5137291 on LNCaP tumors was examined, and it was found that the response period was approximately twice as long as that of bicalutamide.

In conclusion, I established the first cell model of the mechanism of androgen-independent AR activation in CRPC with establishment of the LNCaP-CS10 cell line. Thereby, it was demonstrated that castration resistance was acquired by androgen-independent AR activation, which is the first experimental demonstration of this mechanism (Chapter I). Inhibition of AR nuclear translocation is suggested to

exhibit not only a stronger therapeutic efficacy against CRPCs with AR overexpression, AR mutation, and androgen-independent AR activation as the primary mechanisms of resistance but also against HSPC (Chapter II). I expect that CH5137291 will be further developed in novel therapeutic approaches for prostate cancer.

Besides its effects in prostate cancer, the approach suggested in the present study is expected to also have applications in breast cancer with hormone receptor-dependent proliferation by specifically inhibiting the nuclear translocation of hormone receptors and completely blocking their transcriptional activity. This strategy may exhibit not only a stronger therapeutic efficacy against hormone deprivation therapy-resistant cancers but could also prolong the duration of response to hormone depletion therapy-sensitive cancers.

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Table

Model	Hormone sensitive or Castration resistant	AR status	AR mutation
VCaP	Hormone sensitive	Benchmark	Wild-type
LNCaP	Hormone sensitive	Benchmark	T877A
LNCaP-BC2	Castration resistant	AR overexpression	T877A
LNCaP-CS10	Castration resistant	Androgen-independent activation of AR	T877A
LuCaP35V	Castration resistant	Unknown	Wild-type

Table I.

Castration-resistant prostate cancer models.

Figures

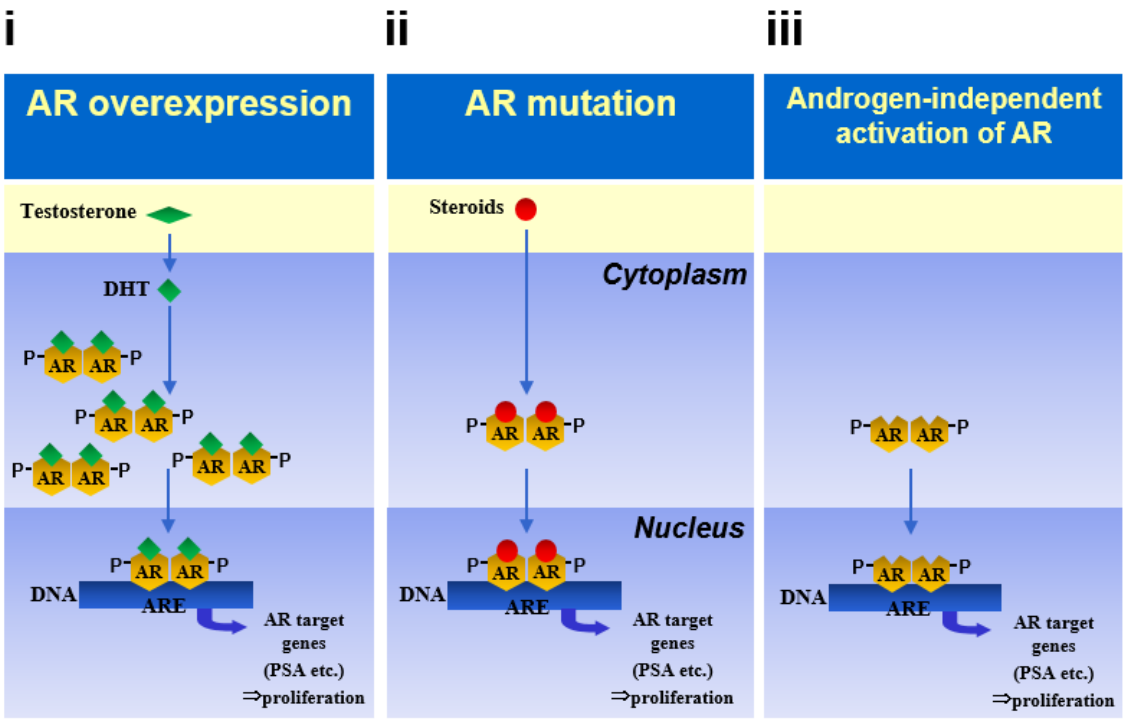

Figure 1.

Mechanisms of castration resistance. i) hypersensitivity to androgen due to overexpression of AR, ii) loss of ligand specificity following an AR mutation, iii) and androgen-independent activation of AR mediated by deregulated growth factors and cytokines.

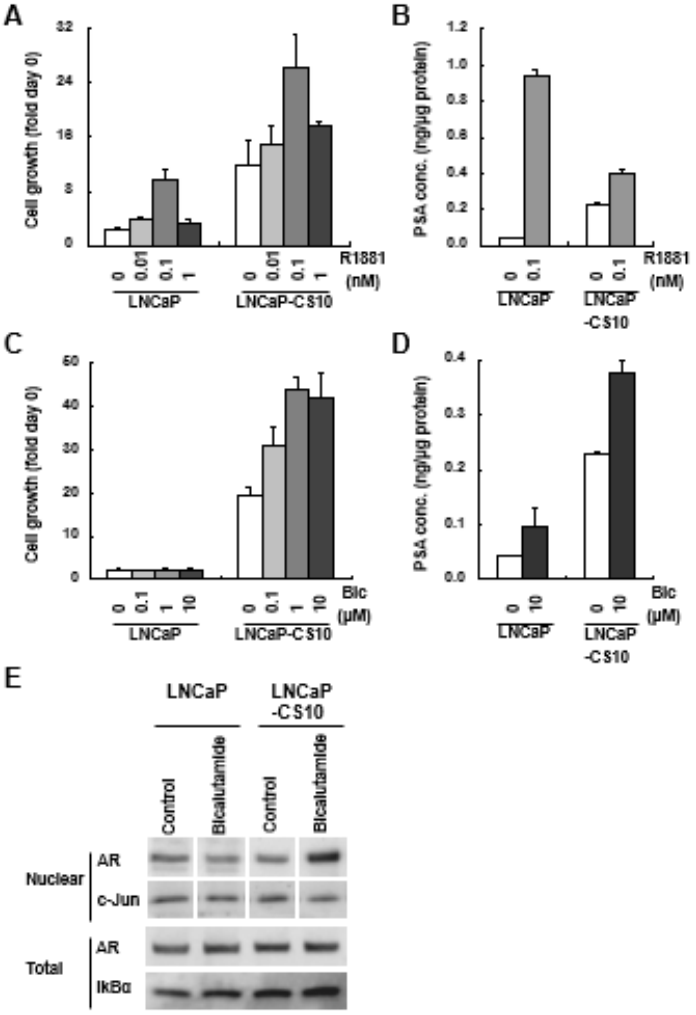

Figure 2.

Effects of androgen and bicalutamide on growth, PSA production, nuclear localization of AR in LNCaP-CS10 cells. (A) Cell growth of LNCaP and LNCaP-CS10 cells was analyzed in the presence of R1881 after 9 days of treatment. (B) Intracellular PSA levels of LNCaP and LNCaP-CS10 cells were measured in the presence of R1881. (C) Cell growth of LNCaP and LNCaP-CS10 cells was analyzed in the presence of bicalutamide after 9 days of treatment. (D) Intracellular PSA levels of LNCaP and LNCaP-CS10 cells were measured in the presence of bicalutamide. All data are expressed as the mean + SD of triplicate determinations. (E) Nuclear localization and expression of AR were determined by Western blotting of AR against nuclear and total fractions, respectively, prepared from LNCaP and LNCaP-CS10 cells in the presence or absence of bicalutamide.

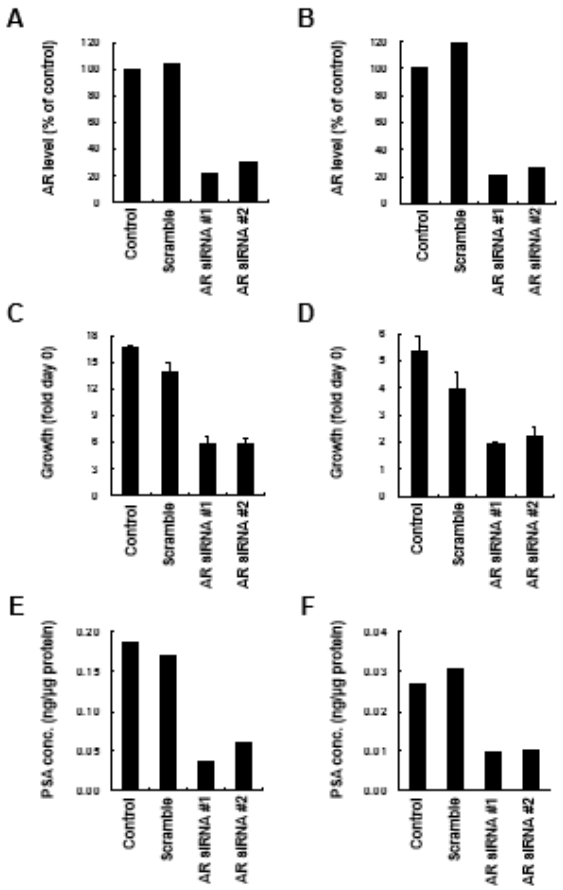

Figure 3.

Effects of AR siRNA on androgen-independent growth of LNCaP-CS10 cells. (A) LNCaP cells were transiently transfected with two AR siRNAs or scramble siRNA. Intracellular AR protein levels were determined by ELISA and are expressed as the percentage of the control (non-siRNA). (B) LNCaP-CS10 cells were transiently transfected with two AR siRNAs or scramble siRNA. Intracellular AR protein levels were determined by ELISA and are expressed as the percentage of the control (non-siRNA). (C) LNCaP cells were transiently transfected with two AR siRNAs or scramble siRNA. Cell count on day 0 and day 6 after transfection was measured and growth is expressed as the percentage of day 0. (D) LNCaP-CS10 cells were transiently transfected with two AR siRNAs or scramble siRNA. Cell count on day 0 and day 6 after transfection was measured and growth is expressed as the percentage of day 0. (E) LNCaP cells were transiently transfected with two AR siRNAs or scramble siRNA. Intracellular PSA levels were determined by ELISA. (F) LNCaP-CS10 cells were transiently transfected with two AR siRNAs or scramble siRNA. Intracellular PSA levels were determined by ELISA. All data are expressed as the mean ± SD of the triplicate determinations.

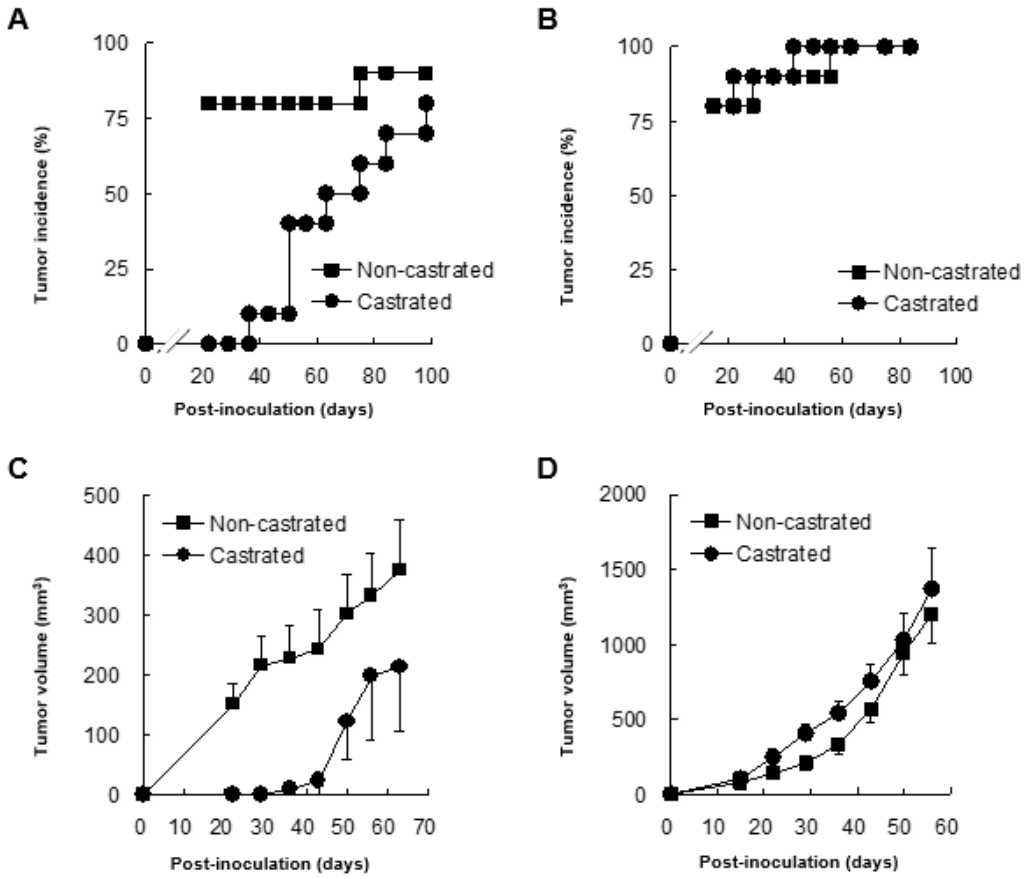

Figure 4.

Tumorigenicity of LNCaP-CS10 in non-castrated and castrated SCID mice. (A) LNCaP cells were inoculated into non-castrated and castrated male SCID mice (*n* = 10/group) and the mice with palpable tumors (>50mm^3) were scored. (B) LNCaP-CS10 cells were inoculated into non-castrated and castrated male SCID mice (*n* = 10/group) and the mice with palpable tumors (>50mm^3) were scored. (C) LNCaP cells were inoculated into non-castrated and castrated male SCID mice (*n* = 10/group) and the tumors were measured. (D) LNCaP-CS10 cells were inoculated into non-castrated and castrated male SCID mice (*n* = 10/group) and the tumors were measured. All data represent the mean ± SE (C and D).

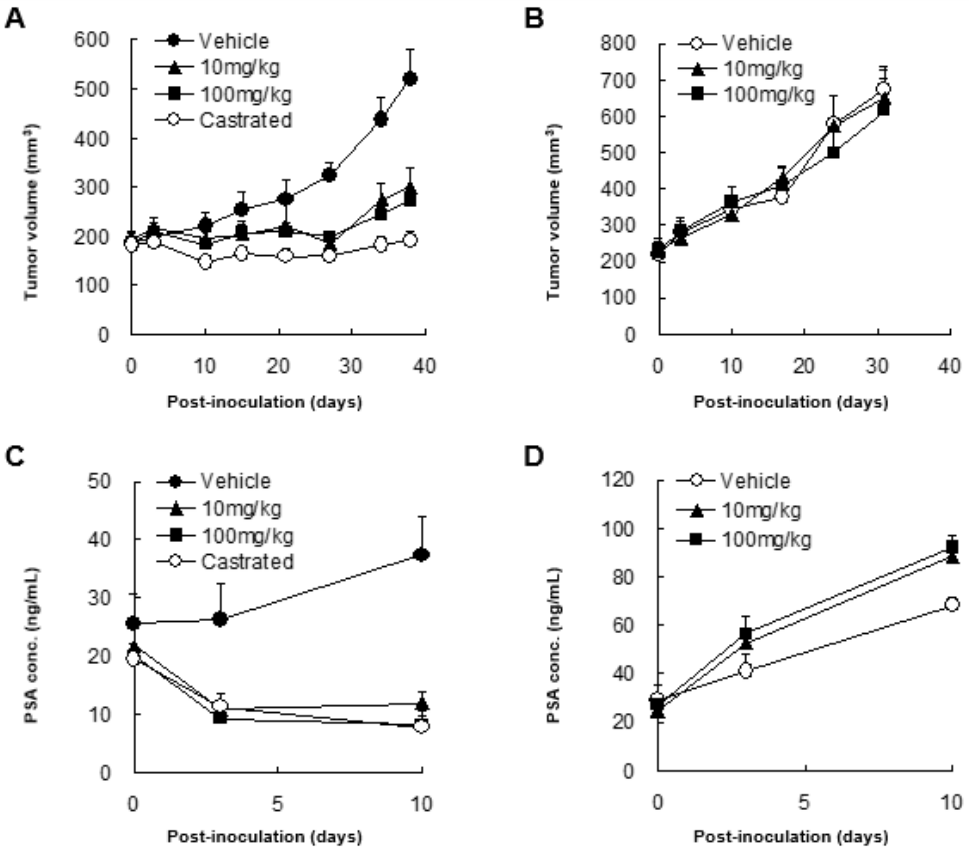

Figure 5.

Effect of bicalutamide on growth of LNCaP-CS10 xenograft tumors and serum PSA levels in castrated male SCID mice. (A) LNCaP cells were inoculated into non-castrated male SCID mice. The tumor-bearing mice were orally administered bicalutamide (0, 10, 100 mg/kg) or castrated (n = 5/group) with administration of the drug for five cycles of 5 days on, 2 days off. Tumor size was measured. (B) LNCaP-CS10 cells were inoculated into castrated male SCID mice. The tumor-bearing mice were orally administered bicalutamide (0, 10, 100mg/kg, n = 5/group) with administration for four cycles of 5 days on, 2 days off. Tumor size was measured. (C) LNCaP cells were inoculated into non-castrated male SCID mice. The tumor-bearing mice were orally administered bicalutamide (0, 10, 100 mg/kg) or castrated (n = 5/group) with administration of the drug for five cycles of 5 days on, 2 days off. Serum PSA levels were measured. (D) LNCaP-CS10 cells were inoculated into castrated male SCID mice. The tumor-bearing mice were orally administered bicalutamide (0, 10, 100mg/kg, n = 5/group) with administration for four cycles of 5 days on, 2 days off. Serum PSA levels were measured. All data represent the mean ± SE.

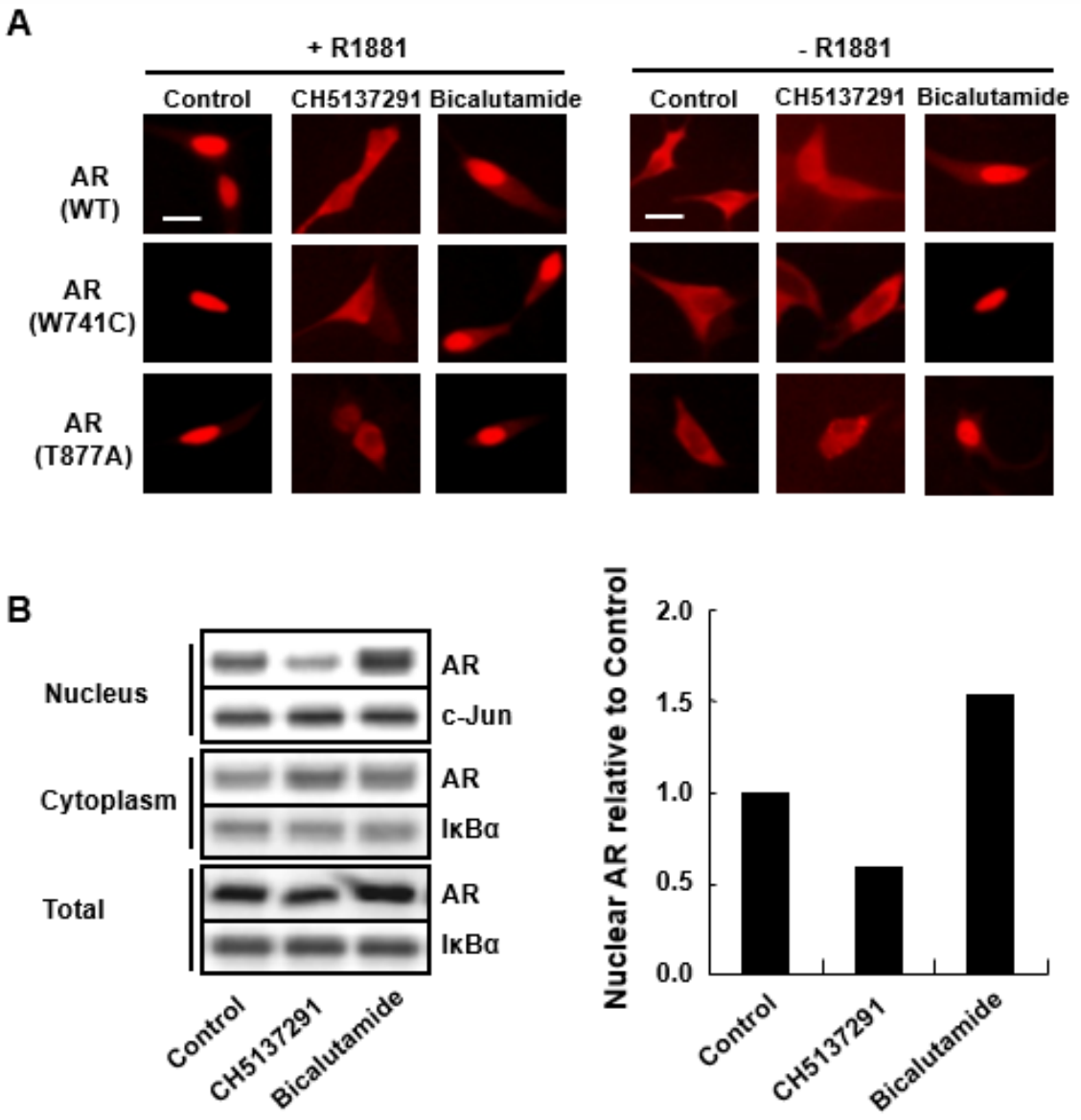

Figure 6.

Effect of CH5137291 on subcellular localization of wild-type and mutant AR. **(A)** Antagonist/agonist effects of CH5137291 and bicalutamide (10 μM) on AR subcellular localization. HaloTag fused with wild-type AR, W741C-mutant AR, or T877A-mutant AR and transiently transfected into LNCaP cells was visualized after 2 h of treatment with CH5137291 or bicalutamide in the presence (0.5 nM) or absence of R1881. Scale bars: 10 μm. **(B)** Effects of CH5137291 and bicalutamide (10 μM) on AR protein levels in nuclear, cytoplasmic, and total fractions of LNCaP-CS10 cells as determined by western blotting in the absence of R1881.

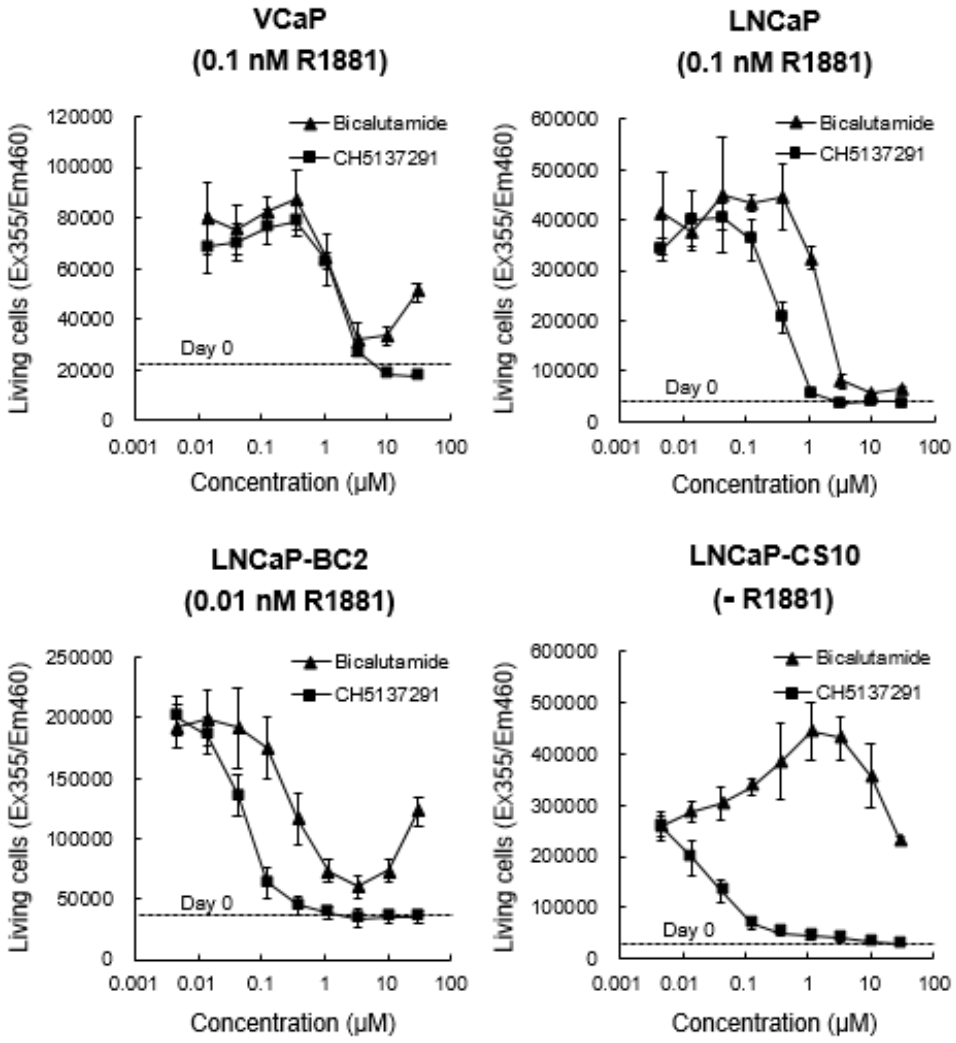

Figure 7.

Growth inhibition of human prostate cancer cells by CH5137291 *in vitro*. Inhibition effects of CH5137291 and bicalutamide on VCaP, LNCaP, LNCaP-BC2, and LNCaP-CS10 cell growth were examined in the presence or absence of R1881 (0.1 nM for VCaP; 0.1 nM for LNCaP; 0.01 nM for LNCaP-BC2; 0 nM for LNCaP-CS10). Living cells on Day 0 are shown by a dotted line. Data represent the mean ± SD of triplicate determinations.

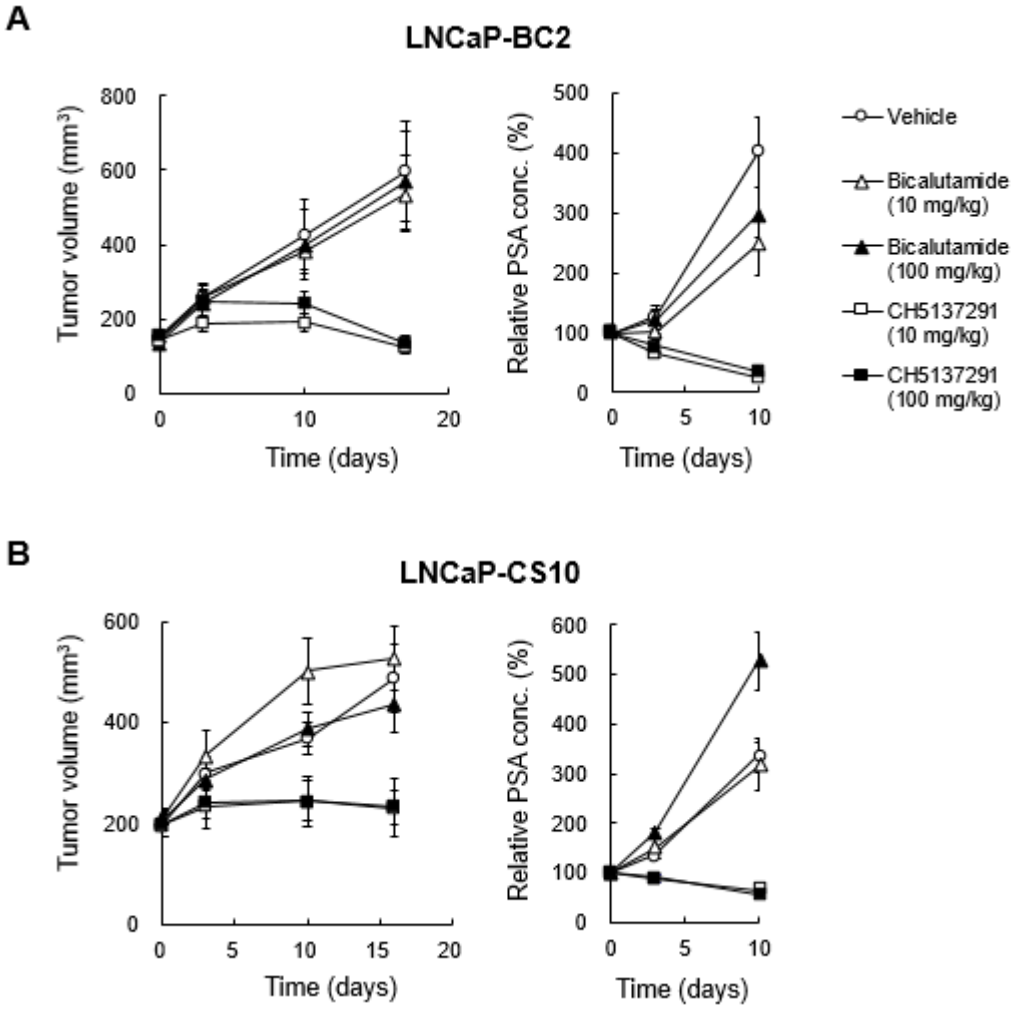

Figure 8.

Antitumor activity of CH5137291 and plasma PSA levels in LNCaP-BC2 and LNCaP-CS10 xenograft models. **(A)** Effects of CH5137291 and bicalutamide (10 and 100 mg/kg) on tumor volume and plasma PSA levels of LNCaP-BC2 xenografts in castrated mice. **(B)** Similar experiments using LNCaP-CS10 xenografts in castrated mice. Data represent the mean ± SE (A, $n = 4$; B, $n = 5$).

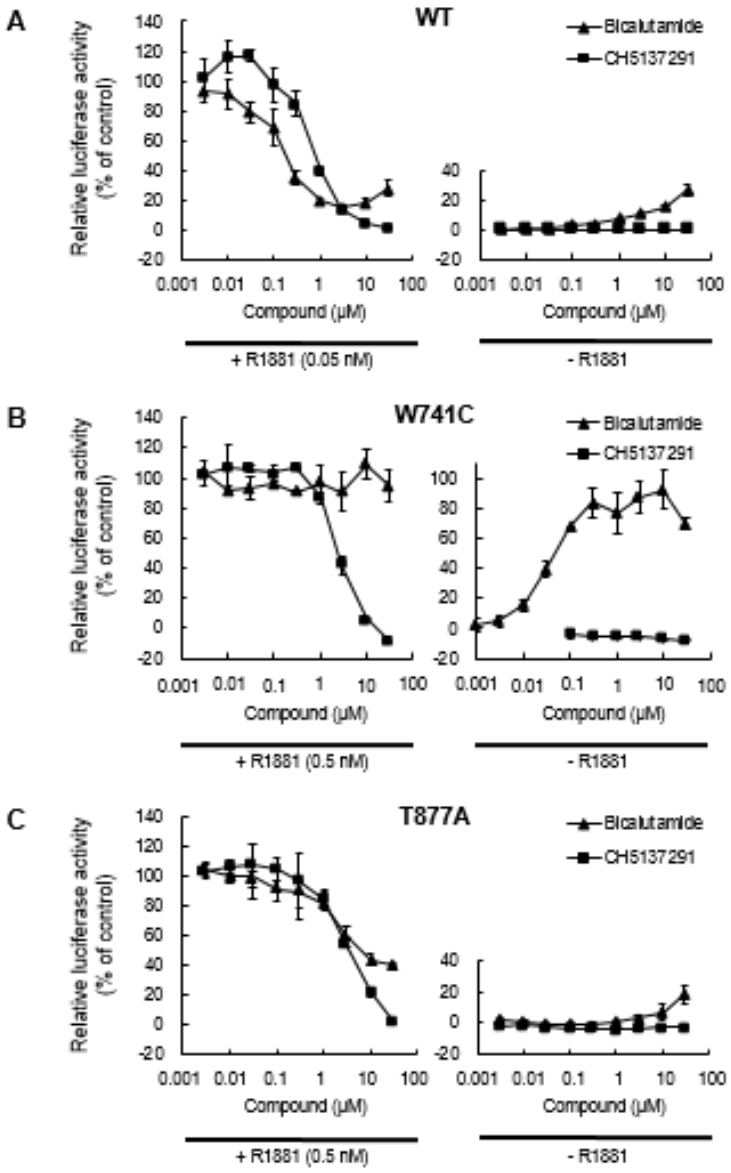

Figure 9.

Effect of CH5137291 on the transcriptional activity of wild-type AR and mutant AR. **(A–C)** The antagonist effect of CH5137291 and bicalutamide on AR transcriptional activity of PC3 cells transiently co-transfected with a reporter vector and the expression vector of wild-type AR **(A)**, W741C-mutant AR **(B)**, or T877A-mutant AR **(C)** was analyzed in the presence of R1881 (0.05 nM for wild-type; 0.5 nM for W741C and T877A). The agonist effect of CH5137291 and bicalutamide was analyzed in the absence of R1881. Data are expressed as a percentage of control (100%: agent is 0 μM and R1881 is as described above; 0%: agent and R1881 are both absent) and the mean ± SD of triplicate determinations.

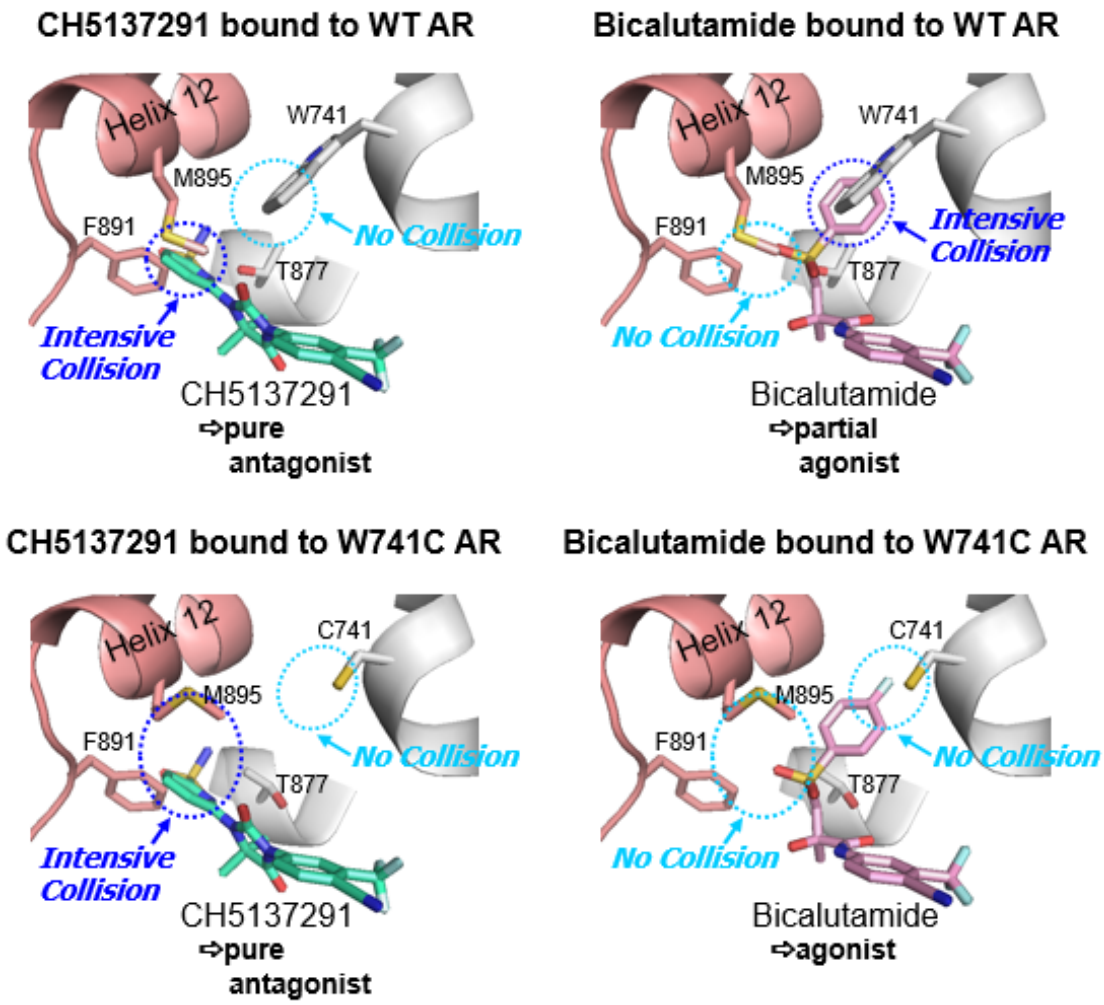

Figure 10.

Docking model of CH5137291 (left) and bicalutamide (right) for wild-type AR (upper) and W741C-mutant AR (lower). W741 residue of wild-type AR (white), C741 residue of W741C-mutant AR (white), M895 residue of wild-type/W741C-mutant AR (salmon pink), CH5137291 (cyan), and bicalutamide (faint pink) are depicted in stick form.

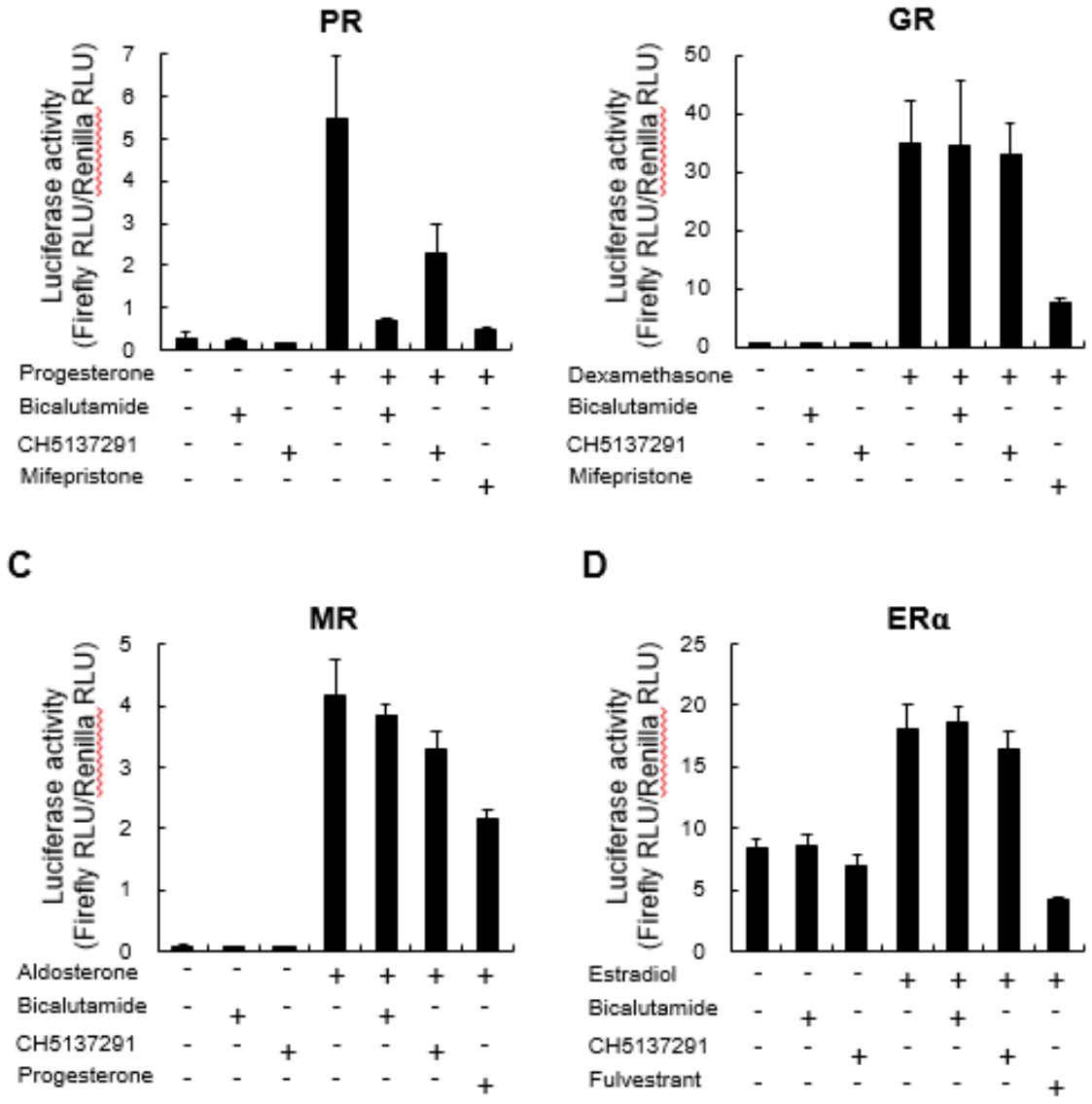

Figure 11.

Nuclear receptor specificity of CH5137291. **(A–D)** Agonist/antagonist effects of CH5137291 and bicalutamide on the transcriptional activity of PR **(A)**, GR **(B)**, MR **(C)**, and ERα **(D)**. Data are expressed as mean + SD of triplicate determinations.

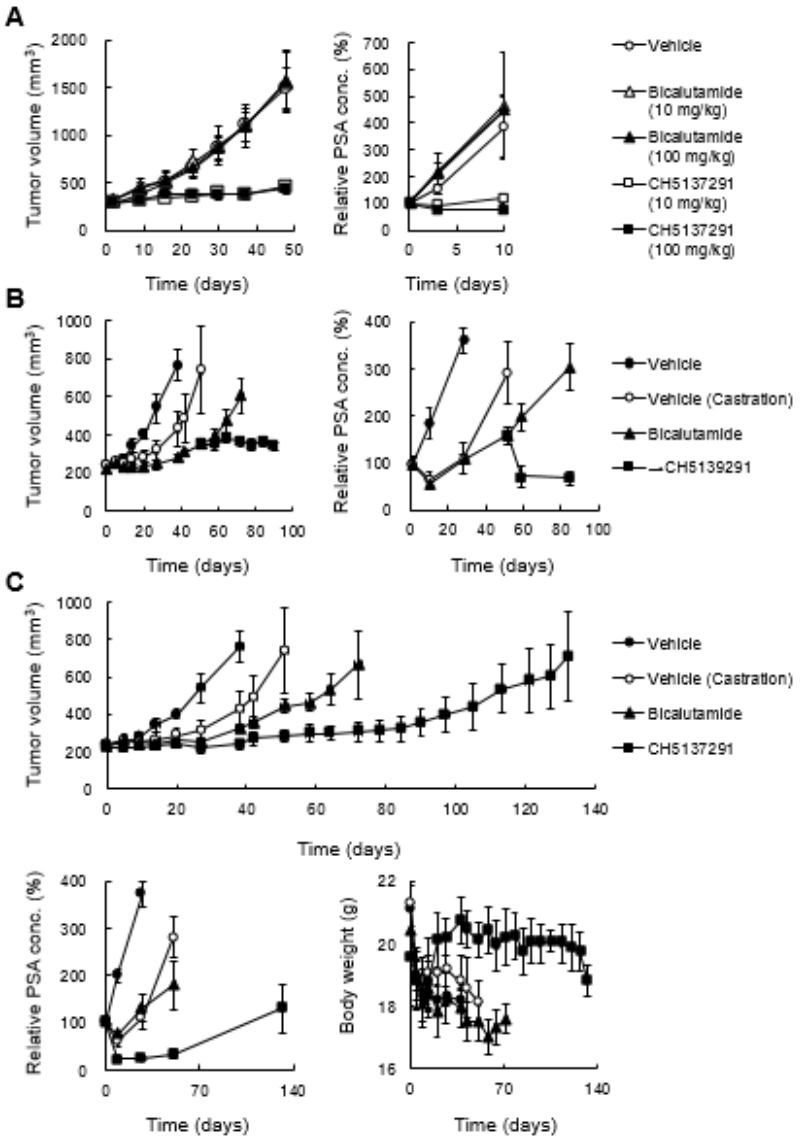

Figure 12.

Antitumor activity of CH5137291 and plasma PSA levels in LuCaP35V and LNCaP xenograft models. **(A)** Effects of CH5137291 and bicalutamide (10 and 100 mg/kg) on tumor volume and plasma PSA levels of LuCaP35V xenografts in castrated mice. **(B)** Effects of switching agent from bicalutamide to CH5137291 on tumor volume and plasma PSA levels. LNCaP tumor-bearing non-castrated mice (n = 25) were administered bicalutamide (100 mg/kg) until a resistant tumor appeared (n = 17). The animals were then randomized and the agent was continued (▲) or switched from bicalutamide to CH5137291 (100 mg/kg) (■). **(C)** Long-term effects of CH5137291, bicalutamide (100 mg/kg), or castration on tumor volume, plasma PSA levels, and body weight of the LNCaP xenograft model in non-castrated mice. All data represent the mean ± SE (n = 5).

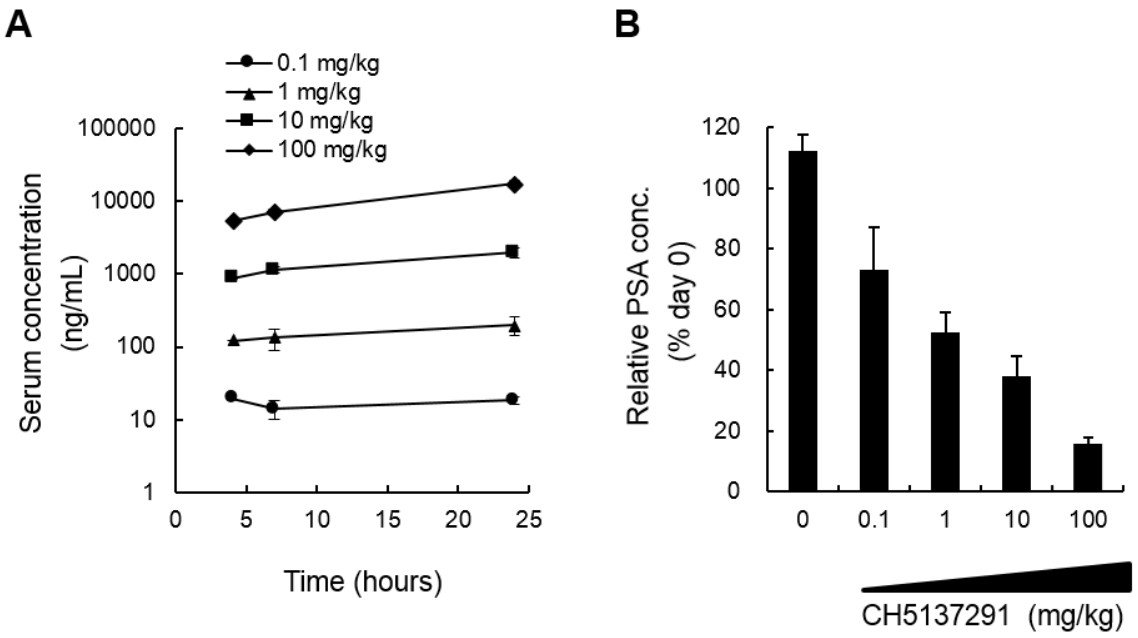

Figure 13.

Dose-dependent exposure of CH5137291 and serum PSA levels in cynomolgus monkeys. **(A)** Analysis of the exposure of orally administered single doses of CH5137291 (0.1, 1, 10, and 100 mg/kg) in male monkeys. Data are expressed as mean ± SE (*n* = 3). **(B)** Serum PSA levels after 7 days of daily administration of CH5137291 (0.1, 1, 10, and 100 mg/kg) in non-castrated male monkeys (*n* = 3). Antagonist activity was determined by the relative PSA concentration normalized to the value on Day 0. Data are expressed as mean + SE.