CH5137291, an androgen receptor nuclear translocation-inhibiting compound, inhibits the growth of castration-resistant prostate cancer cells

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Abstract. Resistance of prostate cancer to castration is currently an unavoidable problem. The major mechanisms underlying such resistance are androgen receptor (AR) over-expression, androgen-independent activation of AR, and AR mutation. To address this problem, we developed an AR pure antagonist, CH5137291, with AR nuclear translocation-inhibiting activity, and compared its activity and characteristics with that of bicalutamide. Cell lines corresponding to the mechanisms of castration resistance were used: LNCaP-BC2 having AR overexpression and LNCaP-CS10 having androgen-independent AR activation. VCaP and LNCaP were used as hormone-sensitive prostate cancer cells. *In vitro* functional assay clearly showed that CH5137291 inhibited the nuclear translocation of wild-type ARs as well as W741C- and T877A-mutant ARs. In addition, it acted as a pure antagonist 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/full agonistic effect on the 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, whereas bicalutamide showed a weaker effect in LNCaP and almost no effect in LNCaP-BC2 and LNCaP-CS10 xenografts. Levels of prostate-specific antigen (PSA) in plasma correlated well with the antitumor effect of both agents. CH5137291 inhibited the growth of LNCaP tumors that had become resistant to bicalu-tamide 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 cynomolgus monkeys, the serum concentration of CH5137291 increased dose-dependently and PSA level decreased 80% at 100 mg/kg. CH5137291 is expected to offer a novel therapeutic approach against major types of castration-resistant prostate cancers.

Introduction

Prostate cancer is common worldwide (1-4). Because most prostate cancers are initially dependent on androgens for their growth, patients with prostate cancer receive androgen deple-tion therapies including surgical castration and administration of luteinizing hormone-releasing hormone analogs. However, in order to achieve total androgen blockade, add-on androgen receptor (AR) antagonists such as bicalutamide (Casodex) or flutamide (Eulexin) are required. Although such total androgen blockade therapy is initially effective (5,6), most patients acquire resistance and progress to more aggressive castration-resistant prostate cancer (CRPC) within several years. Thus, the development of effective therapeutic approaches including the development of more advanced drugs is eagerly anticipated for treating patients with CRPC (7,8).

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 (9). Expression of AR and prostate-specific antigen (PSA) persists in most CRPC specimens (7,8,10), suggesting that the AR signal remains obstinately active. Therefore, AR is still considered to be a potential therapeutic target for CRPC. Pienta and Bradley proposed three major AR-related mecha-

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nisms of castration resistance (Fig. 1) (11): i) hypersensitivity to androgen due to overexpression of AR (12-14); ii) androgen-independent activation of AR mediated by deregulated growth factors and cytokines, termed 'outlaw' activation (15-21); and iii) loss of ligand specificity following an AR mutation (22-26). We have previously reported that existing AR antagonists, such as bicalutamide, have major limitations in that they have a so-called partial agonist profile (27-29). Specifically, these drugs have agonistic activity in addition to the intended antagonistic activity, due to which the drugs show no or at most weak antitumor effect against prostate cancers with castration resistance acquired by the three abovementioned mechanisms (28-30).

It is well known that tamoxifen, an estrogen receptor α (ERα) antagonist widely used against breast cancers, also has a partial agonist profile and tumors can develop resistance to tamoxifen (31). To conquer the problem, ERα pure antagonists, such as fulvestrant (Faslodex), have been introduced and demonstrated to be efficacious against tamoxifen-resistant breast cancers (32-34). In an analogous fashion to ERα, we hypothesized that an AR antagonist without agonistic activity, namely an AR pure antagonist, would be efficacious against CRPC.

Tran *et al* reported a new compound effective against CRPC with AR-overexpression (35). 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 these findings, and taking into account the successful experience with ERα pure antagonists, we screened ~2,000 compounds for their properties as an AR nuclear translocation pure antagonist, and discovered a new compound, CH5137291, showing high-quality properties (36,37).

In the present study, we 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.

Materials and methods

Reagents. CH5137291 and fulvestrant were synthesized in our laboratories. Bicalutamide was purchased as Casodex tablets (AstraZeneca, London, UK) and purified in our 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 our laboratories.

Cells. LNCaP, VCaP, PC3, HeLa, and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). LNCaP-BC2 and LNCaP-CS10 cells were previously established in our laboratory (28,29). LuCaP35V cells that progressed in castrated male mice and expressed wild-type AR were a gift from Professor Robert L. Vessella (University of Washington, Seattle, WA, USA) (38). 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 non-essential 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 (Kanagawa, 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, we 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, IκBα-specific antibody C-15, and c-Jun-specific antibody H-79
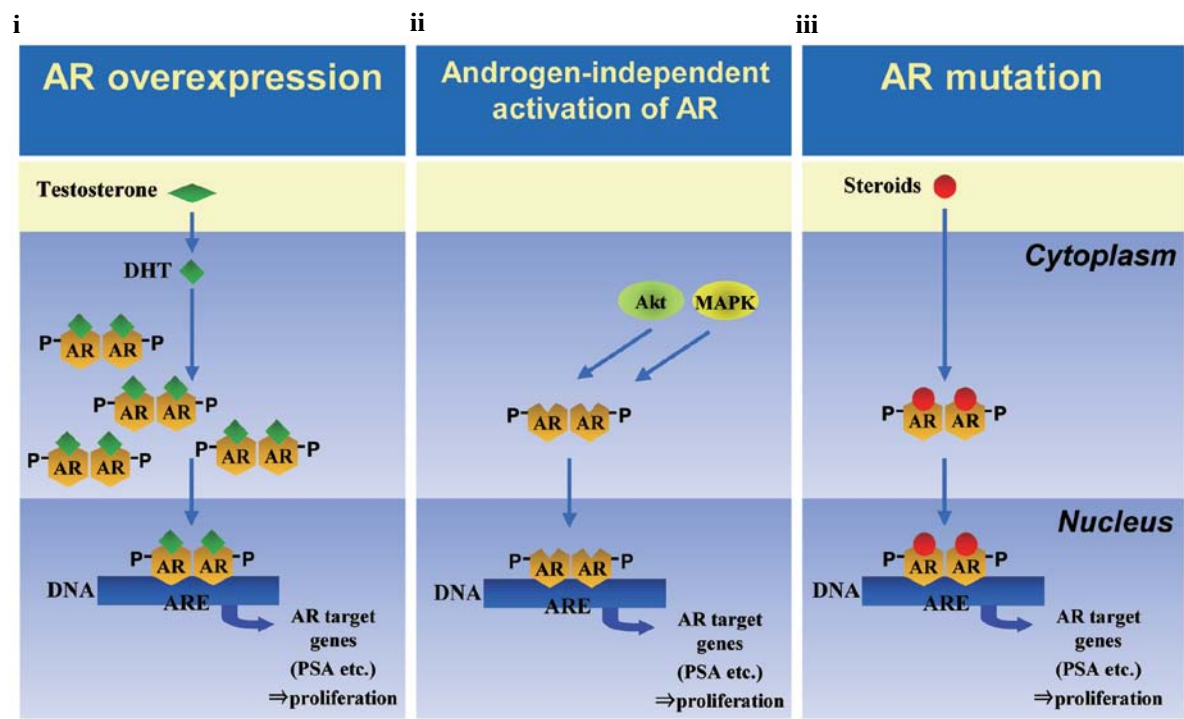
Figure 1. Mechanisms of castration resistance. (i) Hypersensitivity to androgen due to overexpression of AR; (ii) androgen-independent activation of AR mediated by deregulated growth factors and cytokines; and (iii) loss of ligand specificity following an AR mutation.

Table I. Castration-resistant prostate cancer models.

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

(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 2-4 days and were then plated onto poly-D-lysine-coated plates (Becton-Dickinson) (VCaP, $1x10^4$ cells/well; LNCaP, LNCaP-BC2, and LNCaP-CS10, $5x10^3$ cells/well) in 10% DCC-FBS medium. After overnight incubation, CH5137291 or bicalutamide at concentrations of 4.6-30 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-day 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-FBS medium were plated onto 96-well plates at $1x10^4$ 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 progesterone receptor (PR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and estrogen receptor α (ERα). Twenty-four hours before transfection, HeLa cells in 10% DCC-FBS medium were plated onto 96-well plates at $1x10^4$ cells/well for the assays of PR and ERα. COS-7 cells were plated at $0.75x10^3$ 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 model 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 (29). Briefly, LNCaP ($2x10^6$ cells), LNCaP-BC2 ($2x10^6$ cells), LNCaP-CS10 ($2x10^6$ 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 mm³, 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 $TV = ab^2/2$, 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)] x 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) x 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: i) (PSA concentration on day 10 of administration/PSA concentration on day 0 of administration) x 100 < 85%, and ii) 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, we 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* reported the inhibitory effect of CH5137291 on AR nuclear translocation by using COS-7 cells transiently transfected with human wild-type AR (37). In the present study, we examined the effect of CH5137291 on subcellular localization in prostate cancer cell lines with wild-type or mutated
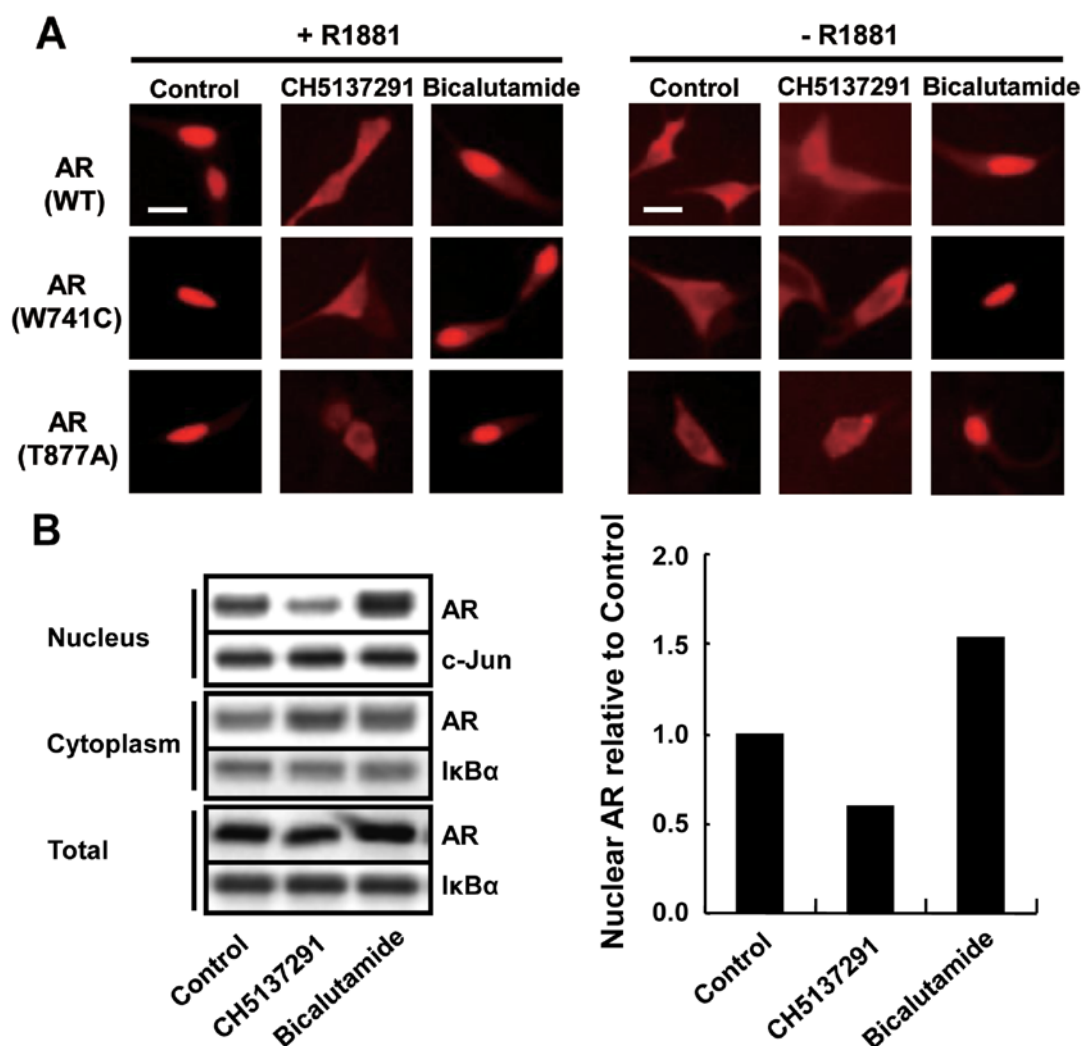
Figure 2. Effect of CH5137291 on subcellular localization of wild-type and mutant AR. (A) Antagonist/agonist effects of CH5137291 and bicalutamide (10 μM) on AR subcellular localization. HaloTag fused with wild-type AR, W741C-mutant AR, or T877A-mutant AR and transiently transfected into LNCaP cells 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.

AR. For this purpose, we 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. CH5137291 in the absence of R1881 did not induce AR nuclear translocation suggesting that CH5137291 does not have an agonistic effect on AR (Fig. 2A). 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, we 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 (29). We found that CH5137291 decreased the nuclear AR level and increased the cytoplasmic AR level without affecting the total AR level (Fig. 2B). 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 and Fig. 3). 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 (29).

In VCaP cells, growth was completely inhibited by CH5137291 at concentrations of 3-30 μM. In contrast, bicalutamide almost completely inhibited the cell growth at 3 μM, however at 30 μM, it induced cell growth. In LNCaP cells,
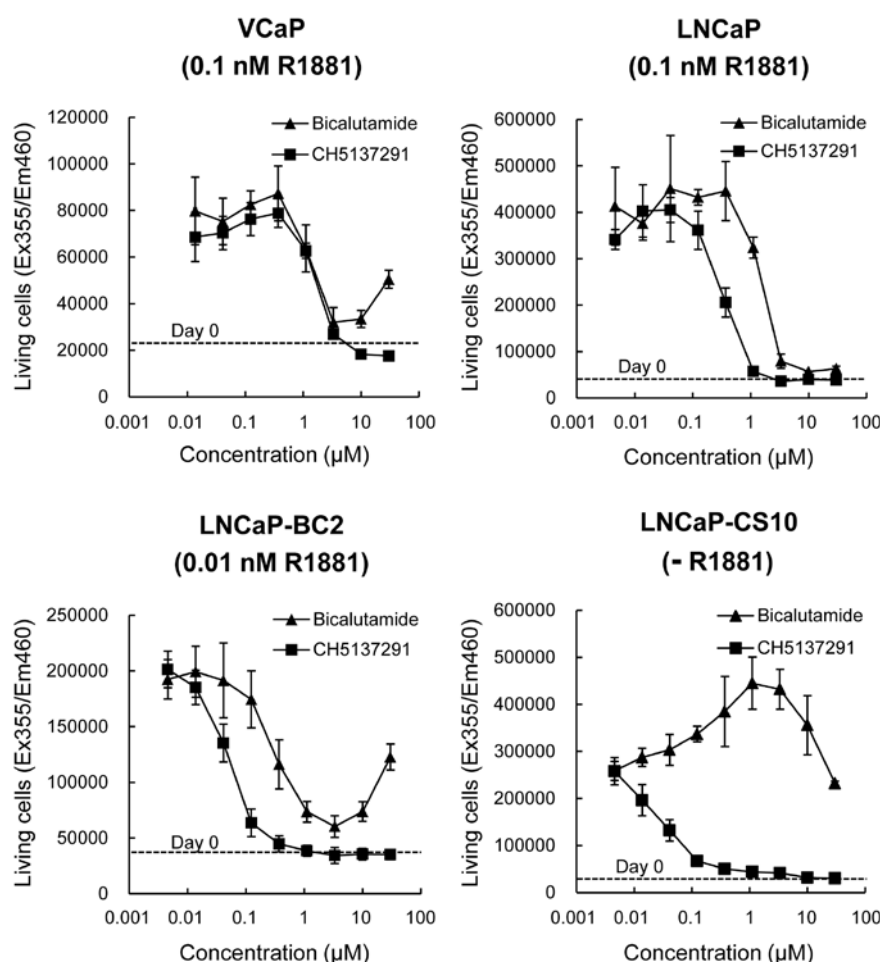
Figure 3. Growth inhibition of human prostate cancer cells by CH5137291 *in vitro*. Inhibition effects of CH5137291 and bicalutamide on VCaP, LNCaP, LNCaP-BC2, and LNCaP-CS10 cell growth were examined in the presence or absence of R1881 (0.1 nM for VCaP; 0.1 nM for LNCaP; 0.01 nM for LNCaP-BC2; 0 nM for LNCaP-CS10). Living cells on day 0 are shown by a dotted line. Data represent the mean ± SD of triplicate determinations.

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. 4). 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. 4). 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 (23,26,30).

In the three transfectants, CH5137291 inhibited transcriptional activity of AR in a dose-dependent manner in the presence of R1881 (Fig. 5). Bicalutamide showed different behaviors in the three transfectants. With wild-type AR, bicalutamide showed dose-dependent inhibition in transcriptional activity ≤3 μM; however, resurgence was observed at higher bicalutamide concentrations (Fig. 5A). With W741C-mutant AR, bicalutamide had no inhibitory effect throughout the concentrations used (Fig. 5B). With T877A-mutant AR, bicalutamide showed a weaker inhibitory effect compared
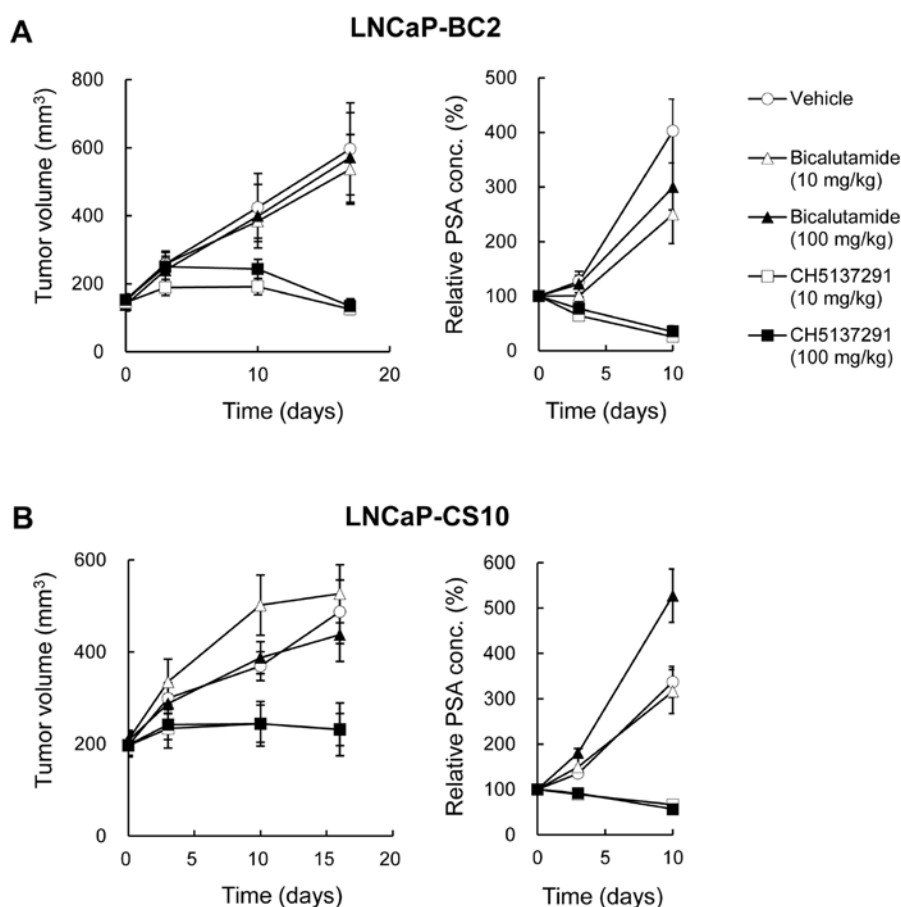
Figure 4. Antitumor activity of CH5137291 and plasma PSA levels in LNCaP-BC2 and LNCaP-CS10 xenograft models. (A) Effects of CH5137291 and bicalu-tamide (10 and 100 mg/kg) on tumor volume and plasma PSA levels of LNCaP-BC2 xenografts in castrated mice. (B) Similar experiments using LNCaP-CS10 xenografts in castrated mice. Data represent the mean ± SE [(A) n=4; (B) n=5)].

with CH5137291. In the absence of R1881, CH5137291 showed no agonistic effect on the transcriptional activity of AR in any of the three transfectants (Fig. 5). In contrast, bicalutamide strongly increased the transcriptional activity of W741C-mutant AR in a dose-dependent manner (Fig. 5B). With wild-type and T877A-mutant AR, bicalutamide increased the transcriptional activity at relatively high concentrations (Fig. 5A and 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 (39,40). 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. 6) (41). 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, we 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 the 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 super-family, we 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 antago-nistic effects on the progesterone receptor as compared to bicalutamide. Moreover, it exhibited neither agonistic nor antagonistic effects on glucocorticoid receptor, mineralocor-ticoid receptor, or estrogen receptor α (Fig. 7).

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 xeno-grafted tumors that had failed to respond to initial bicalutamide
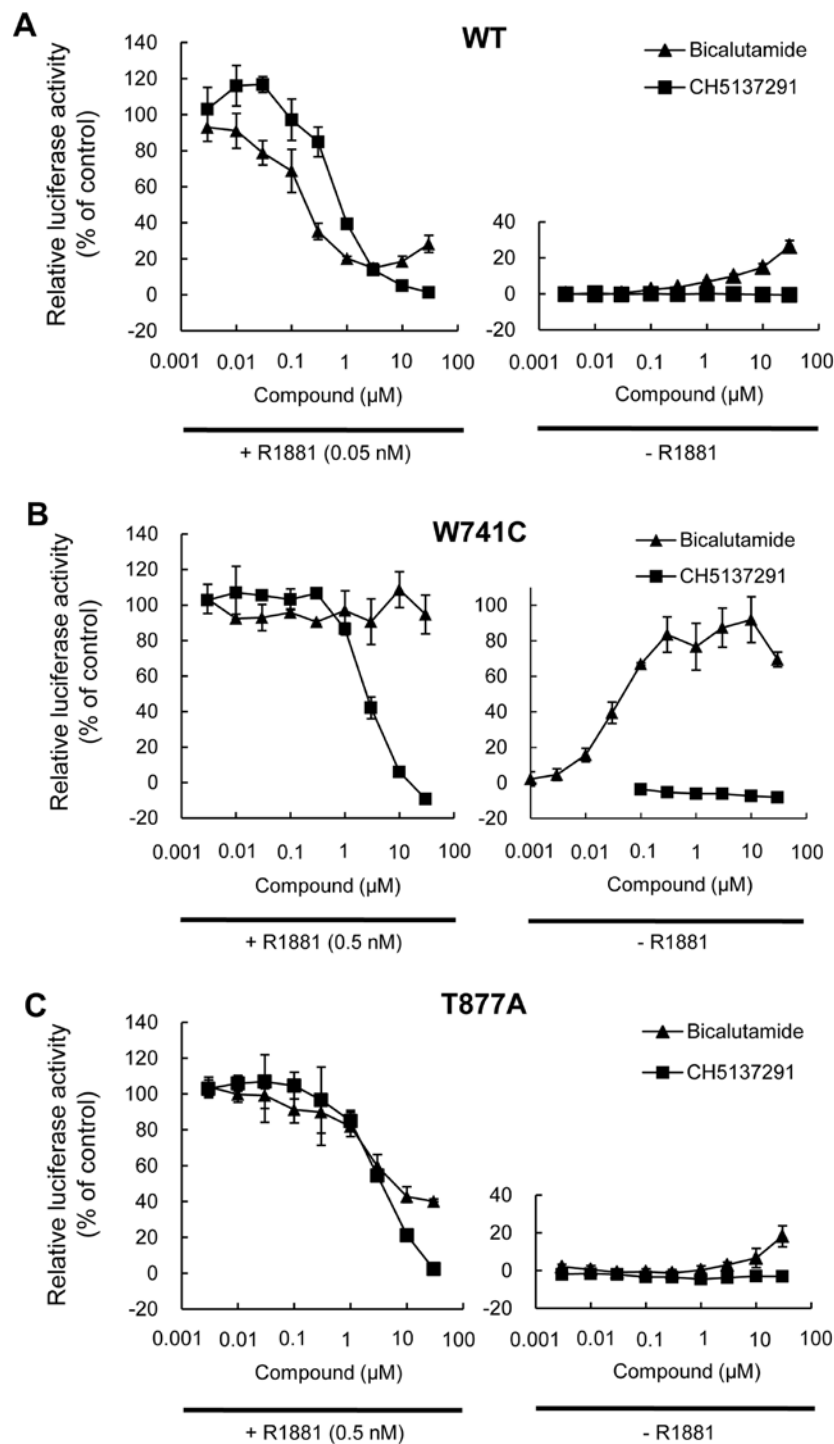
Figure 5. Effect of CH5137291 on the transcriptional activity of wild-type AR and mutant AR. (A-C) The antagonist effect of CH5137291 and bicalutamide on AR transcriptional activity of PC3 cells transiently co-transfected with a reporter vector and the expression vector of wild-type AR (A), W741C-mutant 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.

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. 8A). 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. 8B).

Long-term tumor growth inhibition by CH5137291 in the hormone-sensitive LNCaP xenograft model. Long-term tumor
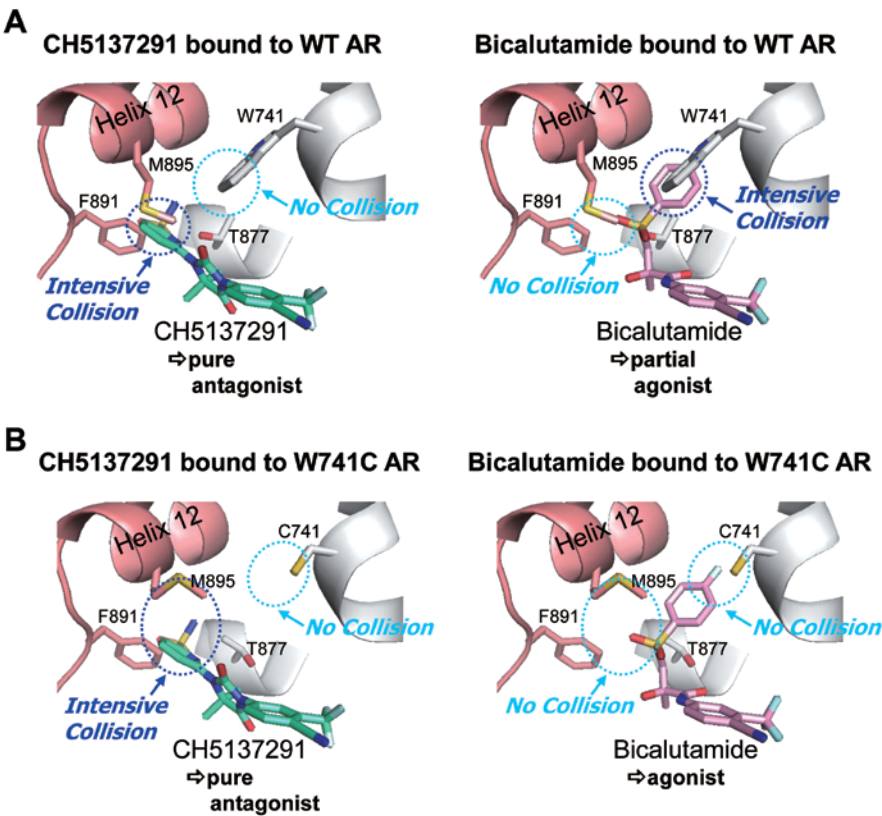
Figure 6. Docking model of CH5137291 (left) and bicalutamide (right) for wild-type AR (A) and W741C-mutant AR (B). W741 residue of wild-type AR (white), C741 residue of W741C-mutant AR (white), M895 residue of wild-type/W741C-mutant AR (salmon pink), CH5137291 (cyan) and bicalutamide (faint pink) are depicted in stick form.
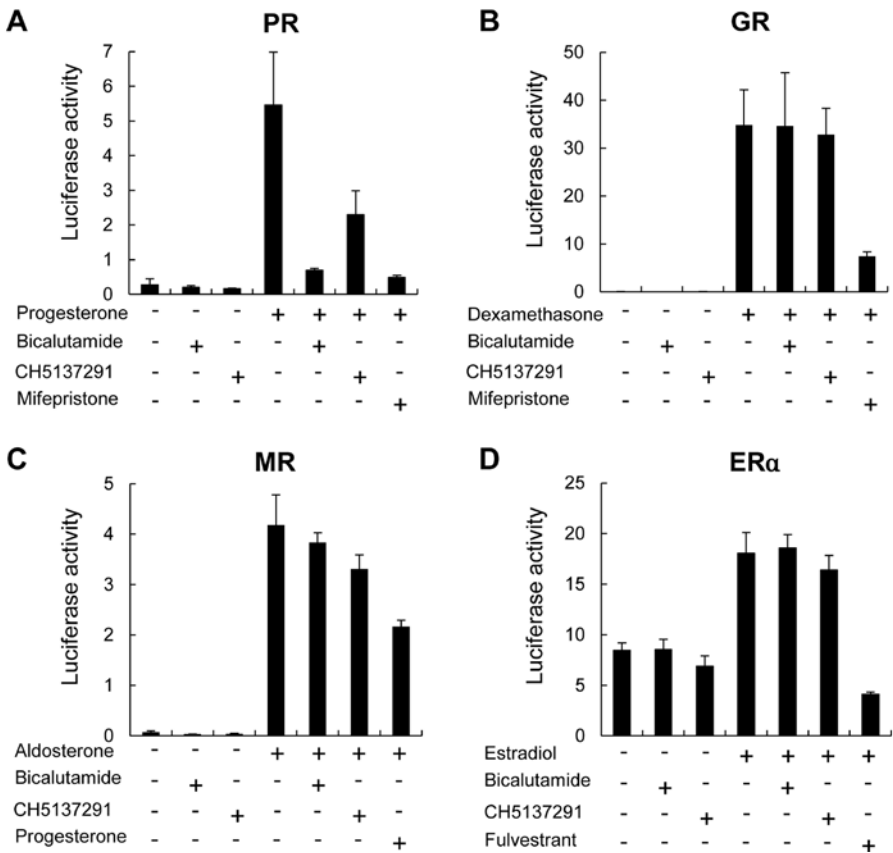

Figure 7. Nuclear receptor specificity of CH5137291. (A-D) Agonist/antagonist effects of CH5137291 and bicalutamide on the transcriptional activity of progesterone receptor (A), glucocorticoid receptor (B), mineralocorticoid receptor (C), and estrogen receptor α (D). Data are expressed as mean + SD of triplicate determinations.
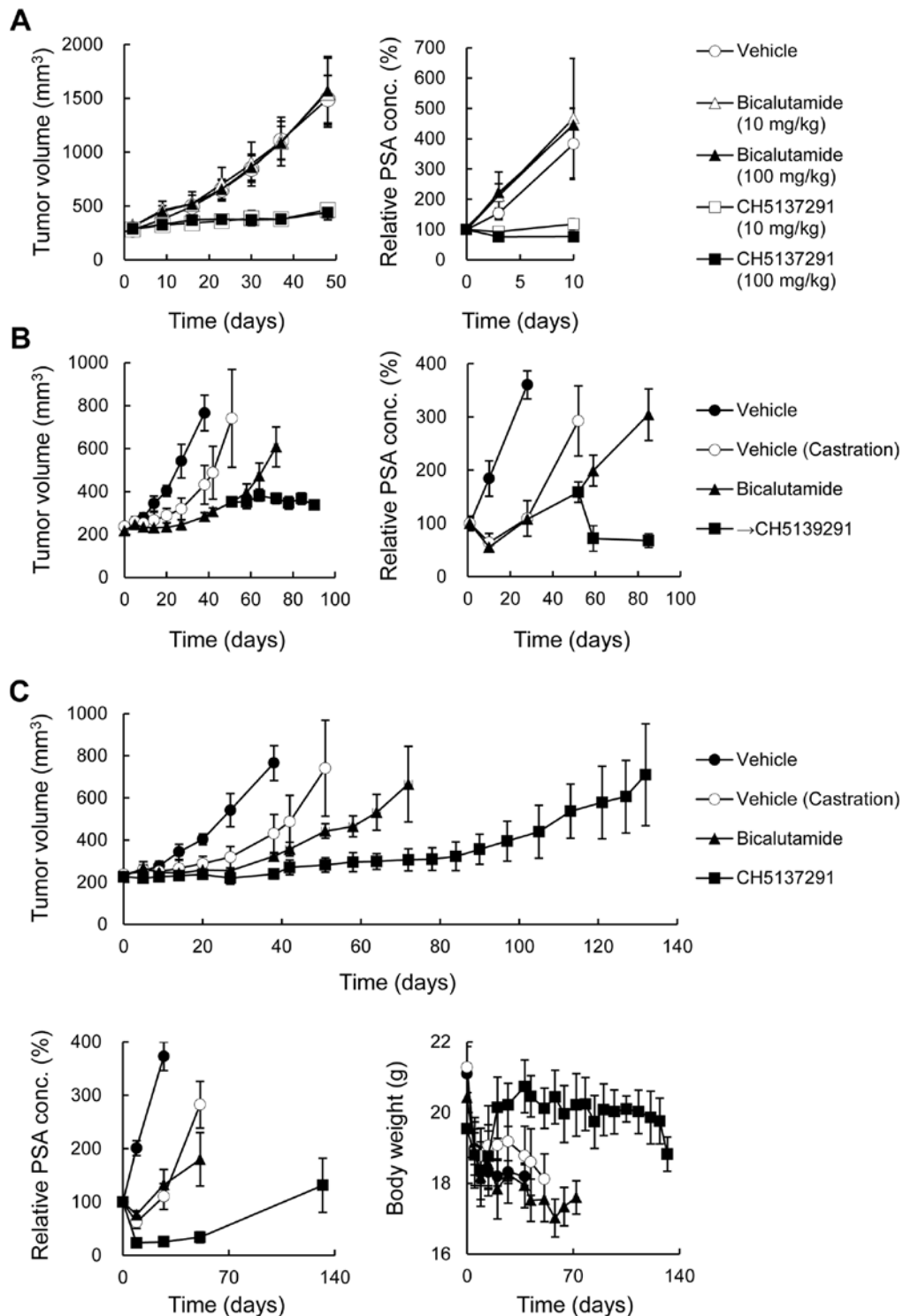
Figure 8. Antitumor activity of CH5137291 and plasma PSA levels in LuCaP35V and LNCaP xenograft models. (A) Effects of CH5137291 and bicalutamide (10 and 100 mg/kg) on tumor volume and plasma PSA levels of LuCaP35V xenografts in castrated mice. (B) Effects of switching agent from bicalutamide to CH5137291 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).

growth inhibition against hormone-sensitive prostate cancer was examined by using an LNCaP xenograft in non-castrated SCID mice (Fig. 8C). 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.
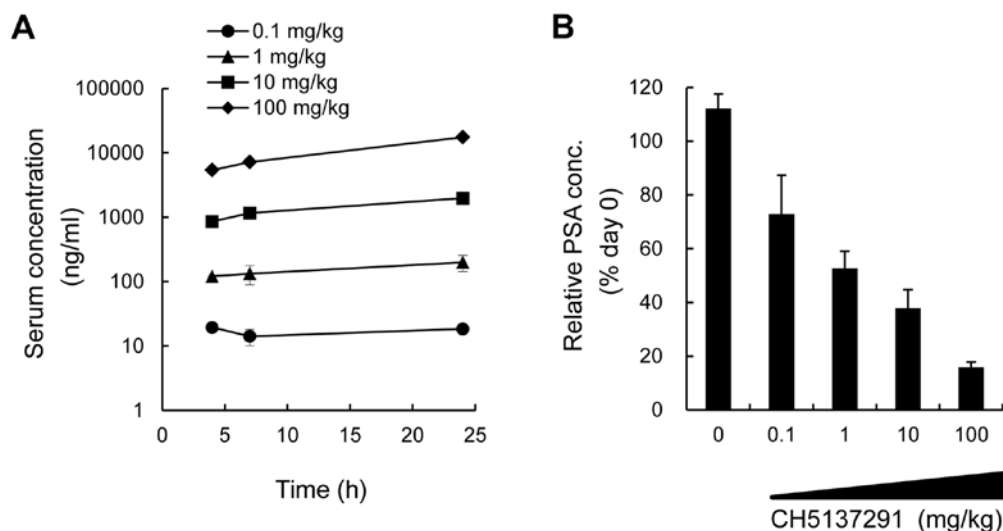
Figure 9. Dose-dependent exposure of CH5137291 and serum PSA levels in cynomolgus monkeys. (A) Analysis of the exposure of orally administered single doses of CH5137291 (0.1, 1, 10 and 100 mg/kg) in male monkeys. Data are expressed as mean ± SE (n=3). (B) Serum PSA levels after 7 days of daily administration 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.

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. 9).

Discussion

Three major AR-related mechanisms of castration resistance in prostate cancer have been reported: i) hypersensitivity to androgen caused by AR overexpression, ii) androgen-independent activation of AR mediated by deregulated growth factors and cytokines; and iii) loss of ligand specificity due to AR mutation (Fig. 1) (11).

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, we focused on the nuclear translocation, and screened for compounds that inhibited nuclear translocation of AR without agonistic activity. We finally found a candidate, the CH5137291. In the present study, we conducted a detailed investigation into the inhibitory activity of CH5137291 on nuclear translocation of AR. We 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. 2A). We also confirmed that CH5137291 inhibited the nuclear translocation of the AR that was endogenously expressed in LNCaP-CS10 (Fig. 2B).

Because the nuclear translocation of AR is required for all three of the abovementioned mechanisms of castra-tion resistance, it was considered that the inhibition of AR nuclear translocation was the most important characteristic of CH5137291.

To investigate the activities of CH5137291 against the three mechanisms of castration resistance, we used 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 (28). LNCaP-CS10 cells proliferate AR-dependently in the absence of androgen *in vitro*, and can grow in castrated male mice (29).

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. 3). In xenograft models of castrated mice with these cell lines, CH5137291 showed significant antitumor activity along with a reduction in plasma PSA levels (Fig. 4). In contrast, bicalutamide did not inhibit tumor growth at all in these xenograft models. These results coincide well with our previous reports (28,29), 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 (Fig. 5) (23,26,30). In contrast, CH5137291 acted as a pure antagonist on the transcriptional activity in each of the ARs (Fig. 5). These results suggest that CH5137291 is potentially superior to bicalutamide

as an AR antagonist against all of the three 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 (39,40). 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. 6). 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 (41). 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.

Recently, reports regarding the development of the CYP17 inhibitor abiraterone acetate and the AR antagonist MDV3100 have described their efficacy against CRPC (35,42-44). Our 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) (29). MDV3100 is reported to have partial agonist activity on AR nuclear translocation (35); 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., progesterone, glucocorticoid, mineralocorticoid and estrogen receptors), any drug targeting AR holds the possibility for cross reactivity-related adverse effects (45,46). Our results indicated that CH5137291 exhibited weaker antagonistic effects on the progesterone receptor as compared to bicalutamide and did not exhibit any agonist/antagonist effects on glucocorticoid receptor, mineralocorticoid receptor, or estrogen receptor α (Fig. 7). We therefore consider that CH5137291 would not cause any adverse events through cross reactivity with other nuclear receptors.

We also examined the effectiveness of CH5137291 in various therapeutic situations. To mimic second-line hormonal therapy, we used a castration-resistant LuCaP35V and LNCaP xenograft models (38). 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. 8A and 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. 8C). These results suggest that CH5137291 was poten-

tially 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 (27,40). 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 [(37) and data not shown] as well as the dose-dependent increase in levels in plasma/serum, which were adequately efficacious not only in mouse xenografts but also in cynomolgus monkeys (Fig. 9). These results suggest that CH5137291 shows promising clinical efficacy.

In conclusion, we have described here the superior characteristics of the novel AR pure antagonist CH5137291 which inhibited AR signaling through the inhibition of AR nuclear translocation. The mechanism of action was the inhibition of helix 12 folding of AR by immediate interference with the M895 residue. Compared with bicalutamide, CH5137291 exhibited not only a stronger therapeutic efficacy against CRPCs with AR overexpression, androgen-independent AR activation, and AR mutation as the mechanisms of resistance but also showed long-term inhibition of growth of hormone-sensitive prostate cancer. We expect that CH5137291 will be involved in novel therapeutic approaches for prostate cancer.

Acknowledgements

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