Characterization of a New Class of Androgen Receptor Antagonists with Potential Therapeutic Application in Advanced Prostate Cancer

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Abstract

The human androgen receptor plays a major role in the development and progression of prostate cancer and represents a well-established drug target. All clinically approved androgen receptor antagonists possess similar chemical structures and exhibit the same mode of action on the androgen receptor. Although initially effective, resistance to these androgen receptor antagonists usually develops and the cancer quickly progresses to castration-resistant and metastatic states. Yet even in these late-stage patients, the androgen receptor is critical for the progression of the disease. Thus, there is a continuing need for novel chemical classes of androgen receptor antagonists that could help overcome the problem of resistance. In this study, we implemented and used the synergetic combination of virtual and experimental screening to discover a number of new 10-benzylidene-10H-anthracen-9-ones that not only effectively inhibit androgen receptor transcriptional activity, but also induce almost complete degradation of the androgen receptor. Of these 10-benzylidene-10H-anthracen-9-one analogues, a lead compound (VPC-3033) was identified that showed strong androgen displacement potency, effectively inhibited androgen receptor transcriptional activity, and possesses a profound ability to cause degradation of androgen receptor. Notably, VPC-3033 exhibited significant activity against prostate cancer cells that have already developed resistance to the second-generation antiandrogen enzalutamide (formerly known as MDV3100). VPC-3033 also showed strong antiandrogen receptor activity in the LNCaP in vivo xenograft model. These results provide a foundation for the development of a new class of androgen receptor antagonists that can help address the problem of antiandrogen resistance in prostate cancer. Mol Cancer Ther; 12(11); 1–11. ©2013 AACR.

Introduction

There is currently no curative treatment for patients with castration-resistant prostate cancer (CRPC) and the corresponding median survival is estimated at ~16 to 18 months (1–3). Numerous studies have shown that the androgen receptor plays a critical, central role in the development and progression of CRPC (4–7) and that inhibition of this nuclear receptor is an effective option for the treatment of late-stage disease.

The currently marketed antiandrogens, such as nilutamide, flutamide, and bicalutamide all act as classical antagonists binding to the hormone binding site (HBS), resulting in conformational changes of the protein which prevent coactivation. Although these drugs initially suppress tumor growth, the cancer often develops treatment resistance. Central among the factors that make the androgen receptor less sensitive to antiandrogens are mutations that weaken the androgen receptor–drug interactions at the HBS. Moreover, certain mutations in the HBS such as W741L/C and T877A convert conventional antiandrogens to agonists which can inadvertently promote cancer growth and progression (8–10). The newly approved drug enzalutamide and investigational drug ARN-509 (11, 12), aim to address this problem, but current reports suggest that resistance to these HBS-directed drugs invariably develops (13). One of the underlying reasons

Note:

Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

H. Li, M.D.H. Hassona, and N.A. Lack contributed equally to this work.

T. Guns’s, P.S. Rennie’s, and A. Cherkasov’s labs made equal contributions to this work.

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is the fact that all known antiandrogens, including enzalutamide, share common structural motifs (Supplementary Fig. S1) responsible for the target binding and, therefore, may be associated with the same risk resistance. Thus, there is a pressing need for non-cross-resistant androgen receptor antagonists with novel molecular scaffolds and/or alternative mechanisms of androgen receptor inhibition.

Materials and Methods

Structure-based virtual screening for novel androgen receptor antagonists

Two docking programs Glide SP (14) and eHiTs (15) were used to dock ~3 million purchasable compounds from the ZINC database (16). Two androgen receptor crystal structures (PDB code:2PNU.pdb and 3L3X.pdb; refs. 17, 18) from the Protein Data Bank (19) were prepared for the docking using Maestro suite (see Supplementary Materials and Methods for details; ref. 20).

Molecular dynamics

We have used the binding pose of the compound VPC-3022 inside of the androgen receptor HBS site generated by docking to run classical molecular dynamics simulation for 10 nanoseconds to evaluate the dynamic behavior of the complex (see details in Supplementary Materials and Methods).

Chemistry

The most common synthetic procedure for the synthesis of benzylidene-10H-anthracen-9-ones is based on the condensation reaction of 10H-anthracen-9-one with aromatic aldehydes. Some of these compounds have been previously reported (21) to act as inhibitors of tubulin polymerization and were obtained as described in that study. Compounds not mentioned therein were synthesized by an aldol-type condensation reaction of 10H-anthracen-9-one with appropriately substituted benzaldehydes under basic conditions in the presence of pyridine/piperidine (see details in Supplementary Materials and Methods). The starting benzaldehydes as well as 10H-anthracen-9-one were obtained from commercial sources.

In vitro studies by cell-based and biochemical assays

Cell culture. LNCaP, LAPC4, and PC3 human prostate cancer cells were obtained from American Type Culture Collection and grown in RPMI 1640 medium supplemented with 5% FBS (Invitrogen). LNCaP, LAPC4, and PC3 cells were tested and authenticated by Idexx Radil (case number 14616-2011) in June 2011. The LNCaP eGFP cell line was stably transfected with an androgen-responsive probasin-derived promoter fused to an eGFP reporter (LN-ARR2PB-eGFP) using a lentiviral approach (22), and were grown in phenol-red-free RPMI 1640 supplemented with 5% charcoal-stripped serum (CSS). HeLa-AR (22), and were grown in phenol-red-free RPMI 1640 supplemented with 5% FBS and 10 μmol/L MDV3100. All cells were maintained at 37°C in 5% CO2.

eGFP cellular androgen receptor transcription assay.
The androgen receptor transcriptional activity was assayed as previously described (22).

Prostate-specific antigen assay.
The evaluation of prostate-specific antigen (PSA) levels secreted into the media was conducted in parallel to the eGFP assay using the same plates. After cells were incubated for 3 days, 150 μL of the media was taken from each well, and added to 150 μL of PBS. PSA levels were then evaluated using Cobas e 411 analyzer instrument (Roche Diagnostics) according to the manufacturer’s instructions.

Biolayer interferometry assay. The direct reversible interaction between small molecules and the androgen receptor was measured as previously described (24).

The androgen displacement assay. The androgen displacement was assessed with the Polar Screen Androgen Receptor Competitor Green Assay Kit (Invitrogen) as per the instructions of the manufacturer.

SRC2-3 peptide displacement assay. The androgen receptor activation function 2 (AF2) specific peptide displacement was assayed as previously described (24, 25).

Transient transfection. HeLa-AR cells were seeded in 96-well plate at a density of 20,000 cells per well in DMEM supplemented with 5% CSS. After incubation for 24 hours, the cells were transfected with ARRS3k-luciferase plasmid (26). Twenty-four hours after transfection, the cells were treated with 0.1 nmol/L R1881 and the tested compounds at different concentrations for 1 day. Then cells were lysed using passive lysis buffer, and assayed for luciferase activity. The luciferase activity was normalized based on the luminescence unit of control wells treated with the vehicle and 0.1 nmol/L R1881 by the formula: %luciferase activity = (sample-vehicle) / (0.1 nmol/L R1881-vehicle) × 100.

Cell viability assay. LNCaP, MDV3100-resistant, and PC3 cells were plated at 3,000 cells per well in RPMI 1640 containing 5% CSS in a 96-well plate, treated with 0.1 nmol/L R1881 and compounds (0–1.5 μmol/L) for 96 hours. After treatment for 4 days, cell density was measured using the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay according to the manufacturer’s protocol (CellTiter 96 Aqueous One Solution Reagent; Promega). The percentage of cell survival was normalized to the cell density of control wells treated with vehicle and 0.1 nmol/L R1881, and calculated as: %survival = (sample-vehicle) / (0.1 nmol/L R1881-vehicle) × 100.

Dose–response Western blot. LNCaP, MDV3100-resistant LNCaP, HeLa-AR, and LAPC4 cells were grown in RPMI 1640/DMEM with 5% CSS (500,000 cells/well) with increasing concentrations of compounds (0–25 μmol/L) in the absence or presence of 0.1 nmol/L R1881. The plates were incubated for 24 hours and then lysed with passive lysis buffer. The protein concentration
was quantified by bicinchoninic acid protein assay kit (Thermo Scientific). Thirty-five micrograms of protein lysate from each treatment was boiled for 5 minutes in sodium dodecyl sulfate (SDS) sample buffer and run on a 12% SDS polyacrylamide gel. The protein was transferred onto a polyvinylidene difluoride membrane. The membrane was probed for androgen receptor with mouse monoclonal AR-441 primary antibody (Santa Cruz) followed by incubation with goat anti-mouse horseradish peroxidase conjugated secondary antibody. As a loading control, β-actin was probed using goat polyclonal antibody, Actin C-11 (Santa Cruz). The proteins were visualized by a chemiluminescent detection system (GE Healthcare).

Quantitative real-time PCR. LNCaP and MDV3100-resistant LNCaP cells were grown in 12-well plates (100,000 cells/well) in RPMI1640 supplemented with 5% CSS. Cells were treated with serially diluted concentrations of compounds (0–25 μmol/L) in the presence or absence of 0.1 nmol/L R1881. Total RNA was extracted from cells using Trizol total RNA isolation reagent. Reverse transcriptase reactions contained 2 μg of RNA samples, 5× first strand reaction buffer, 0.1 M DTT, 10 mmol/L dNTP mix, Random Hexamers, MMLV-RT, and RNase OUT. The reactions (30 μL) were incubated in a 48-well plate for 1 hour at 37°C, 5 minutes at 95°C, and then held at 4°C. Real-time (RT) PCR amplification of cDNA was conducted on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) by mixing 1 μL RT product with 9.5 μL water, 12.5 μL SYBR-GREEN and 1 μL each of the following primers: 5′-GCAGCAAGAGCTGAAGATA-3′ and 5′-CCTTTGTAACCTCCTTCTGA-3′. Androgen receptor gene expression was normalized to 18S rRNA levels in the following injection at time points corresponding to 1, 4, 24 hours. Mice were terminated using CO2 followed by cervical dislocation and blood was immediately collected by cardiac puncture. Typically, 500 to 700 μL of blood was obtained and then placed into a Serum Separator tube (Microtainer; Becton Dickinson), mixed, and placed on ice. Serum samples were centrifuged and the supernatant was placed into eppendorf tubes which were stored at −20°C pending analysis by liquid chromatography/mass spectrometry analysis (LC/MS).

For efficacy studies, 20 6- to 8-week-old nude mice (Harlan Sprague-Dawley, Inc.) weighing 25 to 31 g were subcutaneously inoculated with an LNCaP cell line (10⁶ cells in BD Matrigel; BD Biosciences) at both an anterior and posterior dorsal site. After 28 days, the developing tumors were measured and mice randomly assigned into different treatment groups based on tumor size. Mice-bearing xenograft tumors with volumes between 100 and 200 mm³ qualified for entry into the study (28). Then 3 different treatment groups were assigned: VPC-3033 (10 mg/kg) vehicle (1:10 hydroxypropyl-cyclodextrin:ddH₂O), and enzalutamide. Calipers were used to measure the 3 perpendicular axes of each tumor. The formula \( V = (L \times W \times H) / 2 \) where \( L \) is the length, \( W \) is the width, and \( H \) is the height, was used to calculate the tumor volume. For 3 weeks, 250 to 350 μL volumes of a 1 mg/mL injection solution were intravenously administered via the tail vein twice per week. Mice were also weighed weekly and monitored daily for signs of acute toxicity including death, lethargy, blindness, and disorientation.

Serum extraction and LC/MS. Serum samples generated from the in vivo studies were thawed and transferred to individual eppendorf tubes. Internal standard [5.6 μL of 1 μg/mL deuterated testosterone (d3T, C/D/N Isotopes)] was then added followed by 50 μL of acetonitrile after which samples were vortexed for 5 to 10 seconds and centrifuged as for 5 minutes at 20,000 × g to sediment precipitated protein. The clarified supernatant was transferred to LC vials for analysis. Standards were prepared in a similar fashion using blank mouse serum and also 50% methanol as sample matrix. Optimal grade (Fisher) solvents and 18 MΩ water (Millipore) were used for sample preparation and subsequent LC/MS analysis.

Analysis was carried out with an Acquity UPLC coupled in series with an eLambda PDA and a Quattro Premier (Waters). A 100 mm BEH C18, 1.7μm column (Waters) was used for separations with a 40% to 70% acetonitrile (ACN) gradient from 0.2 to 3 minutes, ramped 1 minute to 95% ACN for flushing (1 minute) followed by 2 minutes re-equilibration for a 7 minute run length (0.1% formic acid present throughout). Wavelengths from 210 to 800 nm at 1.2 nm resolution and 2 points/sec were collected with the PDA. Extracted chromatograms of 375 nm were used for VPC-3033 detection. All MS data were collected in ES+ at unit resolution with the following instrument parameters: capillary, 3.0 kV; extractor...
and RF lens, 5 and 0.1 V; source and desolvation temperatures, 120 and 350°C; desolvation and cone (N2) flow, 900 and 50 L/h; collision gas (Ar) flow, 0.15 mL/min (7.3e−3 m bar). Compounds were detected using multiple reaction monitoring with m/z 299 > 281 for VPC-3033 and m/z 292 > 97 for d3T (24 V/25 V and 32 V/21 V cone/collision volt combinations, respectively) with 0.1-second dwell each. Retention time for d3T and VPC-3033 were 2.55 and 3.8 minutes, respectively.

Quanlynx (Waters) was used for analysis of data with external calibration for PDA data and internal standard normalized calibration for MS data. Calibration standards ranged from 0.01 to 0.9 μmol/L (5 points) with R2 >0.99 and all % deviation from nominal <12% except for OD375 at the lowest concentration. Comparison of spiked serum with neat standards indicated little to no matrix interference, which was present with extraction efficiencies calculated to be 110%.

A major metabolite with retention time of 2.1 minutes was also observed in the OD375 and m/z 299 > 281 channels with a UV/Vis spectrum similar to VPC-3033 and an observed m/z 475 (scan mode), consistent with a glucuronic acid minus H2O adduct. Quantification of this glucuronide was carried out also against the VPC-3033 calibration.

Results

In silico screening for chemically diverse and effective androgen receptor binders

The androgen receptor HBS is primarily composed of hydrophobic residues that can form strong nonpolar interactions with androgenic steroids such as testosterone and dihydrotestosterone (DHT). The protein–ligand anchoring can be additionally stabilized by a network of hydrogen bonds involving R752, Q711, N705, and T877 polar residues. Details of the interactions between androgen receptor and its steroidal and nonsteroidal agonists have been extensively discussed and elaborated (29–31).

However, the mechanism of action of antagonists against androgen receptor is much less characterized, and the relevant structural information is only available for specific mutated forms of the androgen receptor that interact with antiandrogens agonistically (8–10). Combined with the observation that residues forming the androgen receptor HBS are remarkably flexible and can adjust to ligands of various sizes (17, 31), these factors make virtual screening for the androgen receptor a challenging task.

Three million purchasable compounds from the ZINC database (16) were docked into 2 selected crystal structures of the androgen receptor (2PNU and 3L3X) using Glide SP program. About 700,000 compounds that successfully docked with Glide SP score < -7 in both runs were then redocked into the androgen receptor HBS using eHiTs program (15). A total of 130,000 structures were selected that were consistently docked with both algorithms [i.e., having root mean square deviation (RMSD) < 2 Å for the docking poses produced by Glide SP and eHiTs]. These compounds were further evaluated by several onsite rescoring approaches including Glide XP (14), eHiTs score (32), London dG scoring and pKi criteria computed by MOE program (33), and X-score (34).

With this information, a cumulative scoring of 7 different predicted parameters was generated where each molecule received a binary 1.0 score for every “top 10% appearance.” The final cumulative vote (with the maximum possible value of 7) was then used to rank the training set entries. Based on the cumulative score, 50 compounds were selected for experimental evaluation.

Identification of 10-benzylidene-10H-anthracen-9-ones as effective androgen receptor antagonists

The selected 50 chemicals were initially assessed with a nondestructive eGFP reporter assay to quantify levels of androgen receptor transcriptional activity in LNCaP cells, which stably express an androgen-responsive probasin promoter in front of an eGFP reporter (22). Among the tested compounds, 6 compounds showed effective transcriptional inhibition (>85%) when administered at a single 50 μmol/L dose (Table 1). These compounds were then tested for their ability to displace DHT from the androgen receptor using a polar screen competitor green assay kit, and were found to have IC50 values ranging from 0.63 to 50 μmol/L (Table 1). These compounds did not affect SRC2-3 peptide displacement, suggesting they do not interact with the AF2 coactivation site of the androgen receptor (data not shown; ref. 24).

The most active compound VPC-3022 was further measured for the transcriptional inhibition in the eGFP assay at various concentrations, and it showed characteristic dose-dependent behavior, with an IC50 value of 4 μmol/L (Fig. 1A). It is well known that the endogenous androgen receptor in LNCaP cells harbors a T877A mutation. To validate VPC-3022 for its inhibitory efficacy on wild-type androgen receptor, we used HeLa-AR cells transfected with an ARR3 tk-luciferase reporter (26). VPC-3022 showed inhibition of wild-type androgen receptor in a concentration-dependent manner with an IC50 of 1 μmol/L (Fig. S2), consistent with the activity from our eGFP transcriptional inhibition assay in LNCaP cells. To rule out possible false positives from the androgen receptor transcriptional eGFP assay, we validated the results by quantifying the expression of PSA (35). As expected, VPC-3022 induced a dose-dependent decrease of secreted PSA with a corresponding IC50 value of 3.6 μmol/L (Fig. 1A). We have further investigated interaction of the most active compound VPC-3022 with the androgen receptor C-terminal ligand-binding domain (LBD) using the biolayer interferometry (BLI). The BLI results (Fig. 1B) showed direct and reversible interaction between the androgen receptor and VPC-3022 with an equilibrium dissociation constant between compound and protein (KD) value of 4.36 μmol/L. To evaluate the overall effect of VPC-3022 on prostate cancer cell viability, we have conducted the cell viability (MTS) assays using 3 cell lines: LNCaP, PC3 as well as recently developed MDV3100-resistant LNCaP cells. As shown in Fig. 1C, VPC-3022...
exhibit profound concentration-dependent suppression of cell survival, especially with those that have resistance to the current antiandrogen enzalutamide. Although the particular mechanism(s) of MDV3100-resistance in these cell lines has not yet been clearly elucidated, it involves retention of the androgen receptor and no additional receptor mutations (23). More specifically, at a concentration of 1.5 μmol/L, VPC-3022 inhibited the cell proliferation of MDV3100-resistant cells by almost 100% and LNCaP cells by 50%, but only a small effect was observed on androgen receptor-negative PC3 cells.

Interestingly, the VPC-3022 compound was also found to induce an almost complete degradation of the androgen receptor in various prostate cancer cell lines, including HeLa-AR and LAPC4 with wild-type androgen receptor, LNCaP with mutant androgen receptor

![Table 1. Structures and activity profiles of the androgen receptor HBS binders identified from our in silico screening](image)

<table>
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<th>VPC-ID</th>
<th>Structure</th>
<th>% Inhibition at 50 μmol/L (eGFP)</th>
<th>DHT displacement activity IC50 (μmol/L)</th>
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(T877A), and a recently developed MDV3100-resistant LNCaP cells (Fig. 2; ref. 23). At the same time, VPC-3022 did not alter androgen receptor mRNA transcript level (Fig. S3) nor did the addition of the transcriptional inhibitor cyclohexamide affect androgen receptor degradation (Fig. S4), strongly suggesting that the loss of androgen receptor is due to degradation of the protein. The exact mechanism of androgen receptor degradation by VPC-3022 is currently being investigated. Given these results, VPC-3022 was selected as a parental compound for further structural modifications to improve the antiandrogen receptor effect and increase the therapeutic potential.

**Binding mode prediction of VPC-3022 by molecular dynamics study**

To develop an effective strategy for structural optimization of VPC-3022, its predicted interaction with the androgen receptor HBS was investigated in greater details. The docking pose of VPC-3022 reflects mainly a hydrophobic character of its anchoring to the HBS and the overall orientation of the compound in the site resembles positioning of the native ligand—DHT (Fig. S5). Because of the infeasibility of experimental evaluation of antagonistic configurations of the androgen receptor, we have conducted a molecular dynamics simulation of VPC-3022/HBS complex. The established 10 nanoseconds
molecular dynamics trajectories (Fig. S5) reflected overall stability of the complex with a resulting RMSD value around 2.5 Å. The molecular dynamics optimized positioning of VPC-3022 inside the HBS revealed possible formation of a hydrogen bond with residue R752, which had not been captured by the original docking experiment. Based on this predicted binding mode inside the target site, a series of analogues (Table 2) were developed by retaining the anthracenone moiety and modifying the substituents on the benzylidene group.

Development of the 10-benzylidene-10H-anthracen-9-ones as antiandrogen prototype

A series of 10-benzylidene-10H-anthracen-9-ones were synthesized by an aldol-type condensation reaction of 10H-anthracen-9-one with appropriately substituted benzaldehydes under basic conditions in the presence of pyridine/piperidine (Table 2; ref. 36). The created compounds were subsequently evaluated for their ability (i) to displace androgen from the receptor; (ii) to inhibit the androgen receptor in the eGFP and PSA assays; (iii) for their direct interaction with the androgen receptor LBD, as detected by the BLI; (iv) to inhibit the cell proliferation; and (v) to degrade androgen receptor in prostate cancer cells. In these experiments, except a weak agonist (VPC-3037), the rest of tested 10-benzylidene-10H-anthracen-9-ones showed potent androgen displacement from the androgen receptor and effective, dose-dependent inhibition of its transcriptional activity, with corresponding IC50 values estimated in 0.2 to 50 μmol/L range (Table 2). Importantly, 2 particular analogues—VPC-3033 and VPC-3045 showed 10-fold enhanced antiandrogen potency compared with the parental compound VPC-3022. The direct binding to androgen receptor LBD and cell viability were also examined for the analogues, as exemplified by VPC-3033 in Fig. 1, which show similar binding pattern to VPC-3022 and strong inhibition of cancer cell proliferation.

The androgen receptor-degrading ability of these chemicals seemed to be also enhanced—the Western blot images presented in Fig. 2 illustrate almost complete elimination of the receptor from LNCaP, MDV3100-resistant (MDV3100-res.), HeLa-AR, and LAPC4 cells at concentrations of 6 to 25 μmol/L VPC-3033. Of note, 2 other synthesized analogues, VPC-3031 and VPC-3041, also showed significant androgen receptor degradation at concentrations of 6 to 25 μmol/L (Fig. S4). To confirm the androgen receptor degradation by these compounds, the mRNA expression was measured in LNCaP and MDV3100-resistant cells treated at the same concentrations of the compounds as in the Western blot assay. As shown in Fig. S3, the mRNA levels were not affected by the treatment of these compounds at different concentrations. The combined evidence suggests that this class of compounds induces degradation of androgen receptor in prostate cancer cells.

As this chemical class has been reported to act as antimicrotubule agents with tubulin polymerization inhibitory activity (21), the tubulin polymerization activity was
Importantly, as reported previously, the tubulin polymerization inhibitory activity for the highly active androgen receptor inhibitors was not very pronounced, even for the most potent compound VPC-3033 (IC$_{50}$ = 9.9 ± 0.39 μmol/L; Fig. S6), further supporting the conclusion that the observed antiandrogen receptor activity is not simply an artifact as a consequence of the inhibition of tubulin polymerization.

In vivo evaluation of VPC-3033 revealed its ability to suppress androgen receptor function

The lead compound VPC-3033 was investigated for its effect on the tumor growth using the LNCaP xenograft model (28, 37). Results from preliminary acute toxicity studies indicated that doses up to and including 50 mg/kg could be tolerated by the studied mice with no decrease in body weight. The measured serum levels suggested that the compound could be administered effectively via intravenous tail vein injection and that the compound could be detected for up to 24 hours (Fig. 3). At higher doses, we estimate the serum C$_{max}$ to be between 10 and 100 μmol/L. Although clearance was fairly quick, based on in vitro data (Table 2, eGFP IC$_{50}$ = 0.3 μmol/L), the plasma concentration should still be well within the predicted therapeutic window for a substantial duration, up to about 4 hours, given

Table 2. Structures and measured activities of benzylidene-10H-anthracen-9-one derivatives

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Considered very significant.

**Discussion**

Control over a 3-week intravenous dosing regimen (effectively suppressed compared with the vehicle control). At this dose, the growth of tumor was significantly inhibited (12). The dose of 10 mg/kg was chosen based on these preliminary studies and previous literature reports of antagonists.

androgens (12). The 24-hour point of 10 mg/kg was detectable, but beyond quantifiable limitation. B, the in vivo effect of VPC-3033 (10 mg/kg) on tumor volume. The effects of VPC-3033 were determined using LNCaP xenograft model, a tumor xenograft model, a significant effect on the suppression of tumor growth was seen compared with the vehicle and enzalutamide controls (Fig. 3). Although promising, work is currently ongoing to improve the PK/PD characteristics by modifying known sites of potential bioconversion.

Importantly, the chemical scaffold of 10-benzylidene-anthracen-9-one family have been previously implicated in some biological processes including inhibition of tubulin polymerization (21, 38, 39). However, this does not seem to be the mechanism of action observed in this work, as there was no correlation between antitubulin activity and either androgen displacement or androgen receptor transcriptional inhibition.

Previous reports indicated that mutation of W741 to leucine or cysteine will generate additional space in the HBS that allows accommodation of the bulky phenyl ring of bicalutamide and converts its antagonist activity on the androgen receptor into an agonist that stimulates transcriptional activity and cancer growth (40). It has been simulated by docking that binding of VPC-3033 to the androgen receptor HBS occurs at a notable distance from the W741 residue (Fig. 4) and therefore a mutation here is not likely to have an effect on the compound’s androgen receptor binding and activity. Similarly, the well-documented agonist-converting T877A mutation, as found in the androgen receptor

![Figure 3](image-url)

Figure 3. A, a limited PK analysis of VPC-3033 following single 10, 20, and 50 mg/kg i.v. injections displays circulating levels achieved and relatively rapid clearance for both the compound and a major glucuronide metabolite that was observed. C\text{max}, would be expected to be 1 to 2 orders of magnitude higher than the initial 1-hour point collected. The 24-hour point of 10 mg/kg was detectable, but beyond quantifiable limitation. B, the in vivo effect of VPC-3033 (10 mg/kg) on tumor volume. The effects of VPC-3033 were determined using LNCaP mice xenografts (n = 6). Data are presented as mean ± SEM. *, P < 0.05 was considered significant change; **, P < 0.001 was considered very significant change compared with vehicle control.

the PK profile and assuming reasonable absorption. A dose of 10 mg/kg was chosen based on these preliminary studies and previous literature reports of antagonists (12). At this dose, the growth of tumor was effectively suppressed compared with the vehicle control over a 3-week intravenous dosing regimen (P < 0.01 from day 14 onward; Fig. 3). The results clearly indicate that VPC-3033 could effectively inhibit androgen sensitive LNCaP xenograft growth in vivo, suggesting this class of compounds is effective as androgen receptor antagonists.

**Discussion**

As the resistance invariably develops to current antiandrogens in prostate cancer, even to the newly approved second-generation enzalutamide, novel androgen receptor antagonists with new scaffolds and mechanism of action are greatly needed. In this work, a large-scale virtual screening was conducted to find small molecule antagonists for the human androgen receptor that can effectively interact with androgen receptor HBS and yet possess chemical scaffolds that are significantly different from currently used clinical antiandrogens, such as bicalutamide and enzalutamide. Following experimental evaluation of virtual hits, a number of active androgen receptor antagonists were identified in Table 1. Surprisingly, the compound 4-hydroxy-10-benzylidene-10H-anthracen-9-one (VPC-3022) was found to not only effectively interact with the androgen receptor HBS (Fig. 1), but also cause degradation of androgen receptor in prostate cancer cells (Fig. 2).

Following in silico modeling work around the structure of the hit compound (VPC-3022), a 3-hydroxy substituted analogue (VPC-3033) was synthesized (Table 2), characterized, and shown to be a significantly more potent androgen receptor antagonist (Fig. 1). This compound showed a stronger androgen receptor degradation profile (Fig. 2), and exhibited a more profound ability to inhibit the growth of prostate cancer cells. The remarkable inhibitory effect on cells that have developed resistance to the second-generation antiandrogen, enzalutamide (Fig. 1; ref. 11), indicated its potential as a possible second line therapy following the development of resistance to current antiandrogens. When this compound was evaluated in a tumor xenograft model, a significant effect on the suppression of tumor growth was seen compared with the vehicle and enzalutamide controls (Fig. 3). Although promising, work is currently ongoing to improve the PK/PD characteristics by modifying known sites of potential bioconversion.

Importantly, the chemical scaffold of 10-benzylidene-anthracen-9-one is very different from the structures of currently used antiandrogens, which all have a common scaffold (Fig. S1). Chemicals belonging to the 10-benzylidene-anthracen-9-one family have been previously implicated in some biological processes including inhibition of tubulin polymerization (21, 38, 39). However, this does not seem to be the mechanism of action observed in this work, as there was no correlation between antitubulin activity and either androgen displacement or androgen receptor transcriptional inhibition.

Previous reports indicated that mutation of W741 to leucine or cysteine will generate additional space in the HBS that allows accommodation of the bulky phenyl ring of bicalutamide and converts its antagonist activity on the androgen receptor into an agonist that stimulates transcriptional activity and cancer growth (40). It has been simulated by docking that binding of VPC-3033 to the androgen receptor HBS occurs at a notable distance from the W741 residue (Fig. 4) and therefore a mutation here is not likely to have an effect on the compound’s androgen receptor binding and activity. Similarly, the well-documented agonist-converting T877A mutation, as found in the androgen receptor
present in LNCaP cells, should not influence binding of VPC-3033 to this site, as the compound does not form any critical contacts with T877 or its mutant(s) as do flutamide analogues (40). In support of this, our 10-benzylidene-10H-anthracen-9-one derivatives, including VPC-3033, showed effective inhibition of the LNCaP cell line as well as cell lines with wild-type androgen receptor. When tested in the LNCaP xenograft model, the lead compound VPC-3033 showed effective in vivo potency against the tumor growth (Fig. 3).

In conclusion, the established in vitro and in vivo inhibitory activity of VPC-3033 on the human androgen receptor and the prostate cancer cell growth makes this compound an excellent prospective antiandrogen. The preliminary structure–activity relationship information obtained around its analogues may serve as a useful basis for the development of an entirely new class of drugs for treating antiandrogen-resistant prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: H. Li, M.D.H. Hassona, N. Kanaan, K. Singh, H. Adomat, H. Prinz, E.T. Guns, P.S. Rennie, A. Cherkasov
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Li, M.D.H. Hassona, E. Leblanc, P. Tavassoli, K. Frewin, H. Adomat, P.S. Rennie, A. Cherkasov
Study supervision: E. Leblanc, E.T. Guns, P.S. Rennie, A. Cherkasov

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References

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