Medical Research Archives

ESMED
European Society of Medicine

OPEN ACCESS

Published: June 30, 2022

Citation: Amin KS, Hou S, et al., 2022. A Synthetic Small Molecule Carbazole SH-I-125 Degrades Androgen Receptor and Overcomes Castration-Resistance and Enzalutamide-Resistance in Prostate Cancer Cells, Medical Research Archives, [online] 10(6). https://doi.org/10.18103/mra.v10i6.2876

DOI
https://doi.org/10.18103/mra.v10i6.2876

ISSN: 2375-1924

RESEARCH ARTICLE

A Synthetic Small Molecule Carbazole SH-I-125 Degrades Androgen Receptor and Overcomes Castration-Resistance and Enzalutamide-Resistance in Prostate Cancer Cells

Karishma S. Amin[1#], Shu-Jie Hou[2,@], Joshua D. Brown-Clay[1], Milton L. Brown[2,$], and Partha P. Banerjee[1*]

[1] Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, Washington DC.
[2] Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC.
Current address: Health Interactions, 1 Beach St, San Francisco CA 94133
@ Current address: Reyoung Corporation, 704 Quince Orchard Rd, Gaithersburg, MD 20878
$ Current address: George Mason University,10920 George Mason Circle, Manassas, VA 20110.

* ppb@georgetown.edu

ABSTRACT
Resistance to clinical anti-androgens is an ongoing problem in the treatment of castration-resistant prostate cancer (CRPC). Although second generation anti-androgens demonstrate initial clinical benefit, patients often develop resistance to these therapies, evidenced by rising serum PSA levels and disease progression. Several mechanisms including the expression of androgen receptor (AR) splice variants contribute to the reactivation of AR signaling in drug-resistant prostate cancer. Novel therapies that target AR signaling and suppress growth in castration-resistant and anti-androgen-resistant prostate cancer are essential for the effective management of advanced disease. We developed a synthetic small molecule analog of mahanine, SH-I-125, a compound with the ability to disrupt androgen receptor signaling and induce apoptosis in castration-resistant and drug-resistant prostate cancer cellular models. SH-I-125 disrupted AR signaling and induced apoptosis in CRPC cells and anti-androgen-resistant prostate cancer cells in a manner more effective than clinical anti-androgens, enzalutamide and ARN509. Furthermore, SH-I-125 decreased full-length AR and its splice variant AR-V7 levels in 22Rv1 prostate cancer cells by a proteasome-dependent mechanism. These findings indicate the therapeutic potential of SH-I-125 in prostate cancer patients that have progressed on currently approved therapies for CRPC.

Keywords: Androgen Receptor, Apoptosis, Enzalutamide, ARN509, Castration-Resistant Prostate Cancer, Enzalutamide-Resistant Prostate Cancer

Abbreviations used

22Rv1- Castration resistant prostate cancer cell line
AKT: Serine-threonine protein kinase
ANOVA: Analysis of variance
AR: Androgen receptor
ARv7: Androgen receptor splice variant
ARN509: Second generation androgen receptor inhibitor
ARE: Androgen receptor response element
CRPC: Castration-resistant prostate cancer
DHT: Dihydrotestosterone
GR: Glucocorticoid receptor
LBD: Ligand Binding domain
MAPK: MAP kinase
MDV3100: Second generation androgen receptor inhibitor
MDVR: MDV3100-resistant (MDVR)
PARP: Poly adenosine disphosphate-ribose polymerase
PSA: Prostate specific antigen
SEM: Standard error of mean
SH-I-125: Small molecule degrader of AR
STR: Short-tandem repeat
PrEC: primary prostate epithelial cells (PrEC);
RT-PCR: Reverse transcriptase polymerase chain reaction
VCaP: Androgen-dependent prostate cancer cell line

1. Introduction

Prostate cancer is the most commonly occurring non-skin cancer in men, and is estimated to account for 27% of all cancers diagnosed (268,490) in US men in 2022 with 34,500 death.[1] The androgen receptor (AR) signaling pathway is found to be highly active in early- and late-stage prostate cancer. Androgen ablation therapy is commonly used to treat advanced prostate cancer, and is found to be effective in causing initial tumor regression. However, patients who progress on androgen ablation therapy develop a more aggressive androgen-independent cancer phenotype known as castration-resistant prostate cancer.[2] The AR signaling pathway retains activity in patients with castration-resistant prostate cancer in spite of the low levels of circulating androgens. Mechanisms which contribute to the retention of AR activity under castrate conditions include AR gene amplification, mutations that render the AR responsive to other steroids and growth factors, crosstalk with survival pathways such as AKT and MAPK, intra-tumoral and adrenal steroidogenesis, and the expression of constitutively active AR splice variants.[3-9]

Second generation anti-androgens that target the ligand-binding domain (LBD) of the AR have been developed for castration-resistant prostate cancer treatment. Enzalutamide (MDV3100) is currently approved for treatment of patients with metastatic castration-resistant prostate cancer; and its structural analogue, ARN509, is currently under Phase III clinical development. Both *in vitro* and *in vivo* studies showed that both MDV3100 and ARN509 inhibited ligand-binding to the AR. Additionally, both agents decreased the transcriptional activity, target gene expression, nuclear localization and DNA binding of the AR.[10] MDV3100 and ARN509 were effective in preventing the growth of androgen-dependent VCaP cells *in vitro*, and decreased tumor growth in castrate mice *in vivo*. In a Phase III clinical trial in patients that had progressed on chemotherapy, MDV3100 increased overall survival by approximately five months compared to placebo.[11] A recent clinical study showed that MDV3100 decreased the risk of radiographic progression and death in chemotherapy naïve men.[12]

Although clinical data indicate the benefits of MDV3100 therapy, recent evidence indicates the emergence of resistance in castration-resistant prostate cancer cell lines and clinical samples that have undergone prolonged treatment with second generation anti-androgens, MDV3100 and ARN509. MDV3100 is ineffective in preventing the growth of CRPC cell lines like 22Rv1 unless the AR splice variant, AR-V7 is specifically knocked-down.[13] Two independent groups have demonstrated the appearance of a specific missense mutation (F876L) in the AR LBD in cell lines, xenograft tumors, and clinical samples that have undergone prolonged treatment with MDV3100 and ARN509.[14,15] The appearance of this mutation is accompanied by rising PSA levels, indicating resistance to anti-androgen therapy. Additionally, patients who are poor responders to MDV3100 treatment, show increased expression of the glucocorticoid receptor (GR) in bone marrow biopsies.[16] Elevated GR expression has been implicated as a compensatory mechanism to overcome AR antagonism *in vitro* and *in vivo*. Therefore, it appears that the benefits of anti-androgen therapy are short-lived, and alternative approaches to combat emerging resistance are needed for the effective management of castration-resistant prostate cancer.

Since 9H-carbazole was first described in 1872, a number of natural and synthetic carbazoles have been identified as anti-cancer agents and some of them have advanced to various stages of clinical development.[16-18] As an attempt to discover

potent agents for prostate cancer treatment, we found that mahanine (Fig. 1A), a natural carbazole present in the leaves of the curry leaf plant, *Murraya koenigii*, effectively prevented growth and disrupted AR signaling in androgen-dependent and –independent prostate cancer cells. [17] These findings prompted us to synthesize and screen a library of carbazoles to identify a lead candidate that was more effective in disrupting AR signaling. Herein, a novel synthetic carbazole candidate SH-I-125 (Fig. 1A) was identified and found to effectively disrupt AR signaling and induce apoptosis in castration-resistant and MDV3100-resistant prostate cancer cells. Notably, our data showed that SH-I-125 is more effective than both MDV3100 and ARN509. These findings are indicative of the therapeutic potential of SH-I-125 and provide a rationale for further clinical development of SH-I-125 for castration-resistant prostate cancer and anti-androgen-resistant prostate cancer treatment.

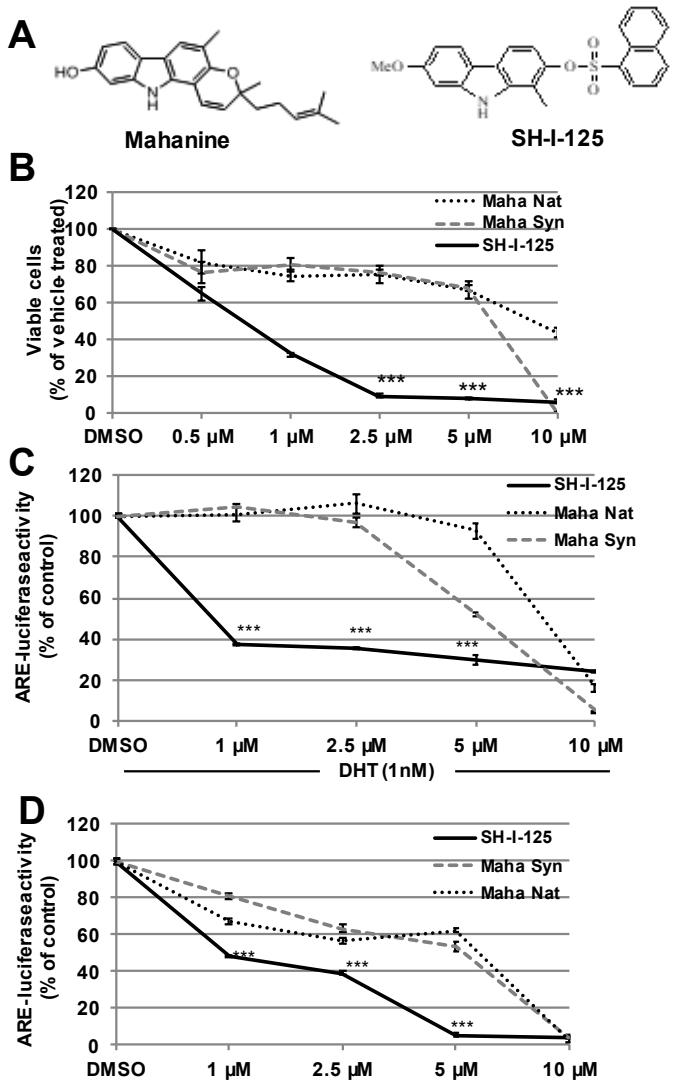


Figure 1: SH-I-125 is more potent than mahanine in preventing prostate cancer cell growth and AR activation
(A) Chemical structure of mahanine (upper panel) and SH-I-125 (lower panel) (B) 22Rv1 cells were treated with the indicated doses of mahanine, synthetic mahanine and SH-I-125 for 3 days. MTT assay was conducted to determine cell viability. The viability of control cells was set at 100%. (C) VCaP-ARE-luc cells were treated with DHT (1nM) in the absence and presence of a range of doses of SH-I-125, natural mahanine (Maha Nat), synthetic mahanine (Maha Syn), for 24 hrs. Luciferase activity was determined and plotted as a percent of control. (D) 22Rv1-ARE-luc cells were treated with a range of doses of SH-I-125, mahanine, synthetic mahanine, for 24 hrs and luciferase activity was determined and plotted as a percent of control. Data represents mean of three independent experiments with quadruplicate samples; bars, SEM. ***, p value <0.001 determined by ANOVA.

2. Materials and Methods

2.1. Synthesis of library of carbazoles including SH-I-125

A library of small molecule carbazoles was synthesized in the Medicinal Chemistry core facility at the Georgetown University Lombardi Cancer Center. A detailed description of the synthesis of SH-I-125 is provided in the Supplementary methods section.

2.2. Reagents

MDV3100 and ARN509 were purchased from Selleckchem and were used in the specified concentrations. DHT (Steraloids, Newport, RI) and forskolin (Sigma, St. Louis, MO) were used in the concentrations indicated below. MG132 was obtained from Tocris Biosciences (Bristol, UK).

2.3. Cell lines

Prostate cancer cell lines (VCaP and CWR22Rv1) were obtained from American Type Culture Collection (Manassas, VA) and were cultured in phenol-red free Improved Minimum Essential Media (IMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM glutamine, 100 U/ml penicillin G sodium, and 100 mg/ml streptomycin sulfate (Sigma, St Louis, MO) unless otherwise specified. PrEC (non-tumorigenic normal primary prostate epithelial cells) were purchased from Lonza (Walkersville, MD) and cultured according to manufacturers' instructions. VCaP cells were cultured in the above media in the presence of DHT (1nM) unless specified otherwise. All cell lines used were tested and authenticated at the Tissue Culture Shared Resource in Lombardi Comprehensive Cancer Center by DNA fingerprinting short-tandem repeat (STR) analysis.

2.4. Establishment of stable VCaP- and 22Rv1-ARE-luciferase cell lines

VCaP and CWR22Rv1 (referred to as 22Rv1) cells were transduced with ARE-luciferase Cignal Lenti Pathway Reporter (Qiagen, Valencia, CA) and cultured as described above in the presence of 1μg/ml puromycin. Following antibiotic selection, cells stably expressing ARE-luciferase were isolated, cloned and cultured to generate stable ARE-reporter cell lines which were used to screen synthetic analogues of mahanine.

2.5. Establishment of MDV3100-resistant VCaP and 22Rv1 cell lines

VCaP cells were cultured as described above, in the presence of DHT (1nM) and MDV3100 (10 μM) for more than 20 passages. Although marginal cell death was noted during early passages, majority of the cells survived, with no cell death occurring in subsequent passages. 22Rv1 cells were cultured as described above, in the presence MDV3100 (10 μM) for more than 20 passages. No cell death was observed, even in early passages.

2.6. Western Blot

Western blotting was performed as described in our previous publication [16]. The following antibodies were used in the concentrations recommended by the manufacturers: Androgen receptor (N-20; Santa Cruz Biotechnology), PSA (A0562; Dako, Carpinteria, CA), β-actin (SC-47778; Santa Cruz Biotechnology), AR-V7 (AG10008; Precision Antibody, Columbia, MD) cleaved PARP (9541), cleaved caspase-3 (9664) (Cell Signaling Technologies, Danvers, MA).

2.7 Nuclear-Cytoplasmic Separation

Cytoplasmic and nuclear fractions of cell lysates were obtained using Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to manufacturer's protocol.

2.8. Immunofluorescence staining

Immunofluorescence staining was performed as described by us previously [17].

2.9. MTT assay

Cells were treated in the manner indicated below for the specified period of time. MTT assay was performed according to a previously published protocol [17].

2.10. Live and Dead cell staining

Live and dead cells were determined according to the manufacturers' instructions (Cell Viability/Cytotoxicity Assay kit, Biotium Inc. Hayward, CA). The live cells stained with calcein AM appeared green and the dead cells stained with EthD-III appeared red.

2.11. Quantitative RT-PCR

The protocol followed for quantitative RT-PCR and the primers used have been previously published [17].

2.12. Caspase Activity Assay

22Rv1 cells were plated in equal numbers in a 96-well plate and treated with SH-I-125, MDV3100 and ARN509 as described below for the indicated periods of time (24-72 hrs), following which caspase 3/7 activity was measured using Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to manufacturer's protocol.

2.13. Statistical analyses

All data were derived from at least three independent experiments and statistical analyses were conducted by using one-way analysis of variance (ANOVA) or Student's t test, where applicable. Values were presented as means ± SEM. *p*-value <0.05 was considered significant.

3. Results

3.1. SH-I-125 is identified an effective disruptor of ligand-induced AR signaling using a cell-based reporter assay

We developed a cell-based assay to screen a library of synthetic small molecule carbazoles and identify potent disruptors of ligand-induced AR signaling with comparable or superior activity to clinical anti-androgens, MDV3100 and ARN509. Androgen-dependent VCaP cells were made to stably express an androgen-response element luciferase reporter (VCaP-ARE-luc). While this system was highly sensitive to DHT stimulation (Suppl. Fig. 1A), it was unresponsive to other steroids (Suppl. Fig. 1B), suggesting that it is specific for AR activation by its ligand.

Upon screening the synthetic carbazoles using VCaP-ARE-luc cells, we identified several candidates that disrupted DHT-induced AR activity in a manner comparable to MDV3100 and ARN509 (Suppl. Fig. 2A-C). Upon further testing, SH-I-125 was identified among these as the most suitable agent for further studies.

3.2. SH-I-125 is more potent than natural carbazole, mahanine in preventing cell proliferation and disrupting AR signaling

Since both SH-I-125 and mahanine are carbazoles, their relative ability to inhibit growth and AR signaling was assessed. SH-I-125 was found to be ten-fold more potent than natural-derived and synthetically-derived mahanine in preventing the growth of androgen-independent 22Rv1 prostate cancer cells (Fig. 1B). Androgen-dependent VCaP cells and androgen-independent 22Rv1 cells stably expressing androgen-response element luciferase (ARE-luc) reporter construct were used to compare the effects of SH-I-125 and mahanine on AR signaling. In both cell lines, SH-I-125 was found to be more potent than mahanine (Fig. 1C and 1D). These data indicate that SH-I-125 is a more potent analogue with greater potential for prostate cancer therapy.

To confirm that SH-I-125 does not ubiquitously and non-specifically inhibit the promoter-luciferase activity of genes, we treated 22Rv1 cells expressing hTERT promoter-luciferase reporter, c-myc promoter luciferase and estrogen response element-luciferase with SH-I-125, and found that luciferase activities of these genes were unaffected (Suppl. Fig. 3A-3C).

3.3. SH-I-125 has inhibitory activity on AR signaling in androgen-dependent cells comparable to second generation anti-androgens, MDV3100 and ARN509

MDV3100 and ARN509 have been repeatedly shown to be potent anti-androgens by competitive inhibition of AR activation by its ligand, DHT. In order to establish whether SH-I-125 could similarly disrupt AR signaling, we treated VCaP cells stably expressing ARE-luc reporter (VCaP-ARE-luc) with DHT (1 nM) in the presence of various concentrations of MDV3100, ARN509 and SH-I-125. Expectedly, MDV3100 and ARN509 effectively inhibited AR transactivation, evidenced by a liner decline in luciferase activity in the presence of increasing amounts of both MDV3100 and ARN509. Treatment with a range of doses of SH-I-125 produced a comparable decline in luciferase activity, suggesting that SH-I-125 possesses the ability to prevent AR activation in a manner similar to MDV3100 and ARN509 (Fig. 2A). Both MDV3100 and SH-I-125 blocked AR nuclear localization, and therefore inhibited PSA expression in VCaP cells (Fig. 2B and 2C), confirming the ability of both agents to inhibit ligand-induced AR transactivation in androgen-dependent cells.

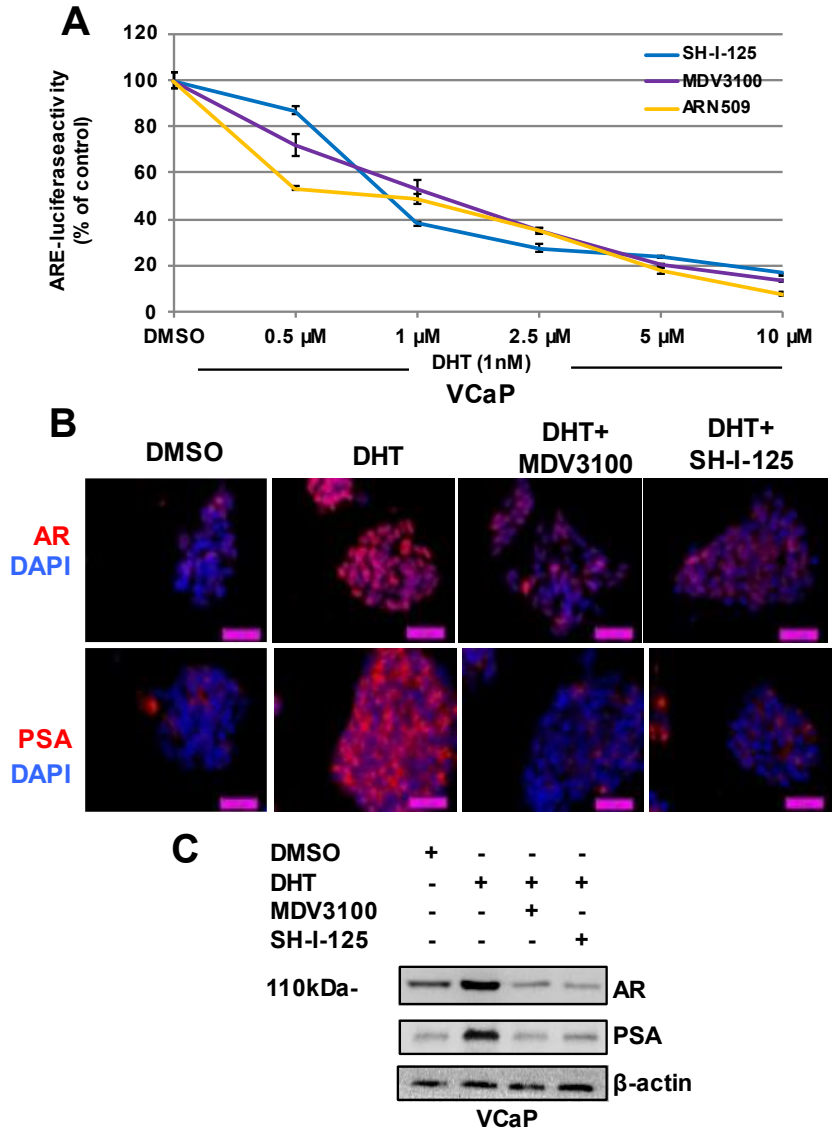


Figure 2: SH-I-125 inhibits AR transactivation in androgen-dependent cells in a manner comparable to clinical anti-androgens

(A) VCaP-ARE-luc cells were treated with indicated doses of SH-I-125, MDV3100 and ARN-509 in the presence of DHT (1nM) for 24 hrs. Luciferase activity was measured as a percent of control. Data represents mean of three independent experiments with quadruplicate samples; bars, SEM. (B) VCaP cells were treated with DHT (1nM) in the absence and presence of MDV3100 (10 μM) or SH-I-125 (1 μM) for 24 hrs. AR and PSA expression levels were assessed by immunofluorescence staining. (C) VCaP cells were treated with MDV3100 (10 μM) or SH-I-125 (1 μM) for 24 hrs in the presence of DHT (1nM). AR and PSA expression was assessed by Western blotting. β-actin was used as a loading control.

3.4. SH-I-125 is a more effective than clinical AR inhibitors, MDV3100 and ARN509, in androgen-independent cells

22Rv1 cells represent a commonly observed castration resistant phenotype, including androgen-independent growth and the expression of AR splice variants such as AR-V7. To determine whether MDV3100 and ARN509 could disrupt AR activation in this cell type, we treated 22Rv1 cells stably expressing ARE-luc reporter (22Rv1-ARE-luc) cells with MDV3100 and ARN509. Interestingly, the inhibitory effect of these anti-androgens plateaued at 50% and did not decrease to baseline, as seen in androgen-dependent VCaP-ARE-luc cells. However, treatment with SH-I-125 was as effective as seen with VCaP-ARE-luc cells, suggesting that SH-I-125 is equally effective in androgen-dependent and androgen-independent prostate cancer cells, and overall SH-I-125 is a more effective AR inhibitor than MDV3100 and ARN509 (Fig. 3A).

Unlike MDV3100 or ARN509, SH-I-125 decreased the nuclear localization of AR and AR-V7 in 22Rv1 cells, further confirming the ability of SH-I-125 to inhibit AR activity in an androgen-independent cell type (Fig. 3B).

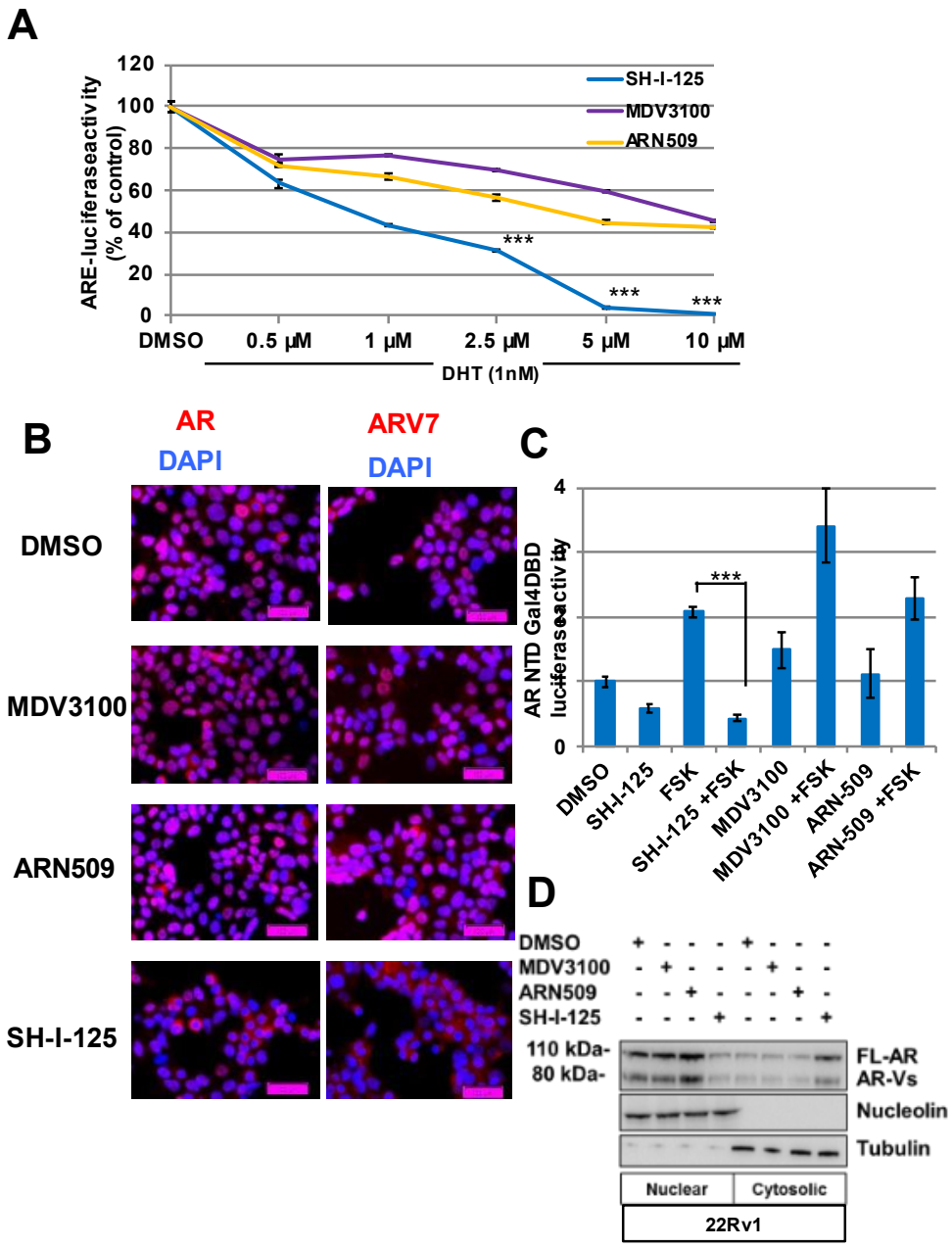


Figure 3: SH-I-125 is a more effective than clinical anti-androgens in preventing AR transactivation in androgen-independent cells

(A) 22Rv1-ARE-luc cells were treated with indicated doses of SH-I-125, MDV3100 and ARN509 in the presence of DHT (1nM) for 24 hrs. Luciferase activity was measured as a percent of control. Data represents mean of three independent experiments with quadruplicate samples; bars, SEM. ***, p value <0.001 determined by ANOVA. (B) 22Rv1 cells were treated with MDV3100 (10 µM), ARN509 (10 µM) or SH-I-125 (1 µM) for 24 hrs. AR and AR-V7 expression was assessed by immunofluorescence. (C) VCaP cells were co-transfected with AR-NTD-Gal4DBD expression vector and Gal4UAS-TATA-luciferase reporter construct and treated with forskolin (FSK) (50µM) in the absence and

presence of SH-I-125 (1 μM), MDV3100 and ARN509 (10 μM) for 24 hrs. Luciferase activity was measured after normalization with Renilla luciferase. ***, p value <0.001 determined by ANOVA.

The AR-NTD is known to be its transactivation domain, essential for ligand-independent AR activation. Our previous report [17] demonstrated the ability of mahanine to inhibit AR activation induced by ligand-independent mechanisms such as forskolin and interleukin-6, which activate the AR N-terminal domain (NTD).[18, 19] In VCaP cells, which were made to co-express an AR NTD-Gal4-DNA binding domain (AR NTD-Gal4DBD) fusion protein and a luciferase reporter containing the Gal4 binding site (UAS-Luc), SH-I-125 significantly inhibited forskolin-induced AR activation (Fig. 3C). As a negative control, we co-expressed a constitutively active VP16-Gal4DBD fusion protein and the UAS-luc reporter in VCaP cells and found that SH-I-125 did not affect luciferase activity, suggesting that its effects on the AR NTD are independent of any effect on the Gal4DBD (Suppl. Fig. 4). Interestingly, MDV3100 and ARN509, which specifically target the AR LBD, were found to increase forskolin-induced AR activation by an unknown mechanism (Fig. 3C). These results indicate that SH-I-125 is effective in disrupting ligand-independent activation of the AR, but not clinical anti-androgens, MDV3100 and ARN509.

3.5. SH-I-125 inhibits growth in CRPC cells, but not in non-tumorigenic primary prostate epithelial cells

To determine the effects of SH-I-125 on prostate cancer cell growth, we treated 22Rv1 cells with SH-I-125 measured cell viability after a period of 3 days. We found that at a dose of 1μM, cell viability was reduced to 50% of control in the presence of SH-I-125, while MDV3100 and ARN509 were significantly less effective in preventing the growth of 22Rv1 cells (Fig 4A). In comparison, non-tumorigenic primary prostate epithelial cells (PrEC) retained 80% viability in the presence of a similar range of doses of SH-I-125, suggesting that tumorigenic cells are more susceptible to the growth inhibitory properties of SH-I-125 compared to non-tumorigenic cells (Fig. 4B and Suppl. Fig. 5A and 5B). In addition, upon staining to specifically detect live and dead cells upon SH-I-125 treatment, we found that although SH-I-125 killed 22Rv1 cells, PrEC were mostly unaffected, in spite of the five-fold higher dose used to treat PrEC compared to 22Rv1 (Suppl. Fig. 6A and 6B). Staurosporine was used as a positive control for cell death induction in live and dead assay (Suppl. Fig. 6B). Together, these data confirmed that SH-I-125 has differential cytotoxic effects on tumorigenic vs non-tumorigenic cells.

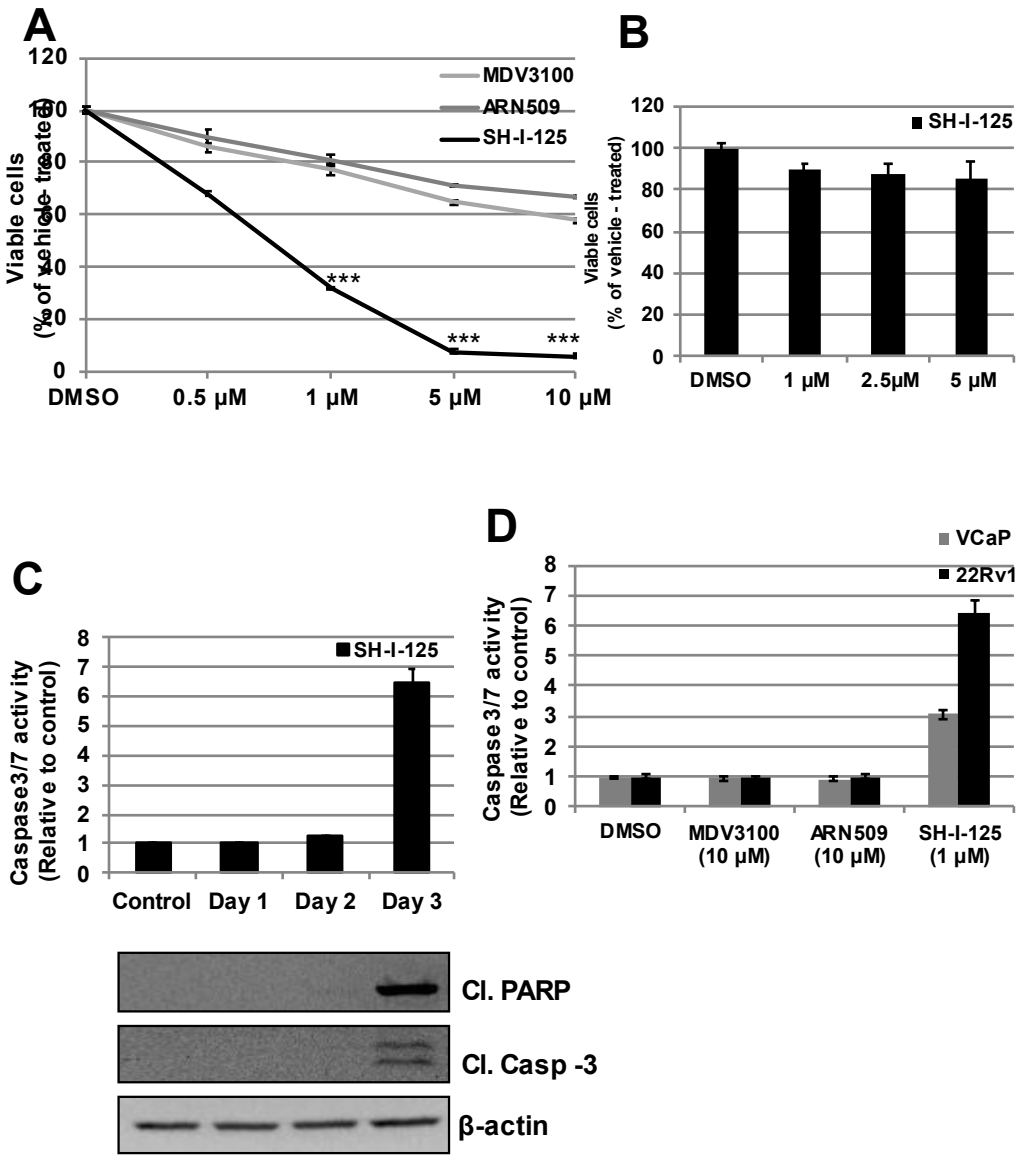


Figure 4: SH-I-125 inhibits growth and induces apoptosis in androgen-independent prostate cancer cells
(A) 22Rv1 cells were treated with the indicated doses of SH-I-125 for 3 days and (B) PrEC cells were treated with the indicated doses of SH-I-125 for 3 days. MTT assay was conducted to determine cell viability. (C) 22Rv1 cells were treated with SH-I-125 (1 µM) for one, two or three days, and caspase 3/7 activity was measured using Caspase-Glo 3/7 substrates. (D) 22Rv1 cells were treated as described in (C) and cells were harvested to assess levels of cleaved caspase-3 and cleaved PARP by Western blotting. β-actin was used as a loading control. (D) VCaP and 22Rv1 cells were treated with MDV3100, ARN509 and SH-I-125 for 3 days. Caspase 3/7 activity was measured using Caspase-Glo 3/7 substrates. Data represents mean of three independent experiments with quadruplicate samples; bars, SEM. ***, p value <0.001 determined by ANOVA.

3.6. SH-I-125, but not MDV3100 and ARN509, induces caspase-mediated apoptosis in prostate cancer cells

The incidence of elevated cell death in the presence of SH-I-125 led us to explore whether caspase activity and apoptosis were induced in 22Rv1 cells upon SH-I-125 treatment. A time-course of SH-I-125 treatment revealed that caspase-3 and -7 activities were significantly induced in 22Rv1 cells after three days treatment. Caspase activation was confirmed by the presence of cleaved caspase-3 and accompanied by the cleavage of PARP, a well-known target of caspase-3, after three days of SH-I-125 treatment (Fig 4C and Suppl. Fig. 7). Staurosporine was used as a positive control for apoptosis induction (Suppl. Fig. 6B)

While SH-I-125 induced caspase activity in both androgen-dependent VCaP cells and androgen-independent 22Rv1 cells, MDV3100 and ARN509 did not have a similar effect in either cell lines, suggesting that the second generation anti-androgens are ineffective in inducing caspase-mediated cell death in androgen-independent prostate cancer cells (Fig. 4D).

3.7. SH-I-125, but not MDV3100 and ARN509, reduces cellular levels of AR and AR-V7 by proteasomal degradation

To determine whether SH-I-125 affected the cellular levels of the AR and its splice variants, we treated 22Rv1 cells with a range of doses of SH-I-125 for 3 days, and 1μM SH-I-125 for 1-3 days (Fig. 5A). Additionally, 22Rv1 cells were treated with a range of doses of MDV3100 and ARN509 for a period of 3 days (Fig. 5B and 5C). While MDV3100 and ARN509 had no effect on AR and AR-V7 levels, SH-I-125 reduced AR and AR-V7 levels at a dose of 1μM in a period of 3 days, indicating that in addition to inhibition of AR activity, SH-I-125 was effective in declining the cellular levels of the AR and its splice variant.

To establish a mechanism by which SH-I-125 caused a decline in AR and AR-V7 levels, we measured the relative expression of AR and AR-V7 in 22Rv1 cells that had been treated with SH-I-125 for a period of 1-3 days. Our data shows that SH-I-125 did not affect the message levels of these genes, suggesting that AR and AR-V7 decline post-translationally in cells treated with SH-I-125 (Fig. 5D). To determine whether proteasomal mechanisms are responsible for the decline in AR and AR-V7 levels, we treated 22Rv1 cells with 5 μM SH-I-125 for a period of 48 hrs in the presence of proteasome inhibitor, MG132. Proteasome inhibition rescued the decline in AR and AR splice variant levels in the presence of SH-I-125 (Fig. 5E), suggesting that SH-I-125 induces proteasomal degradation of AR and its splice variant.

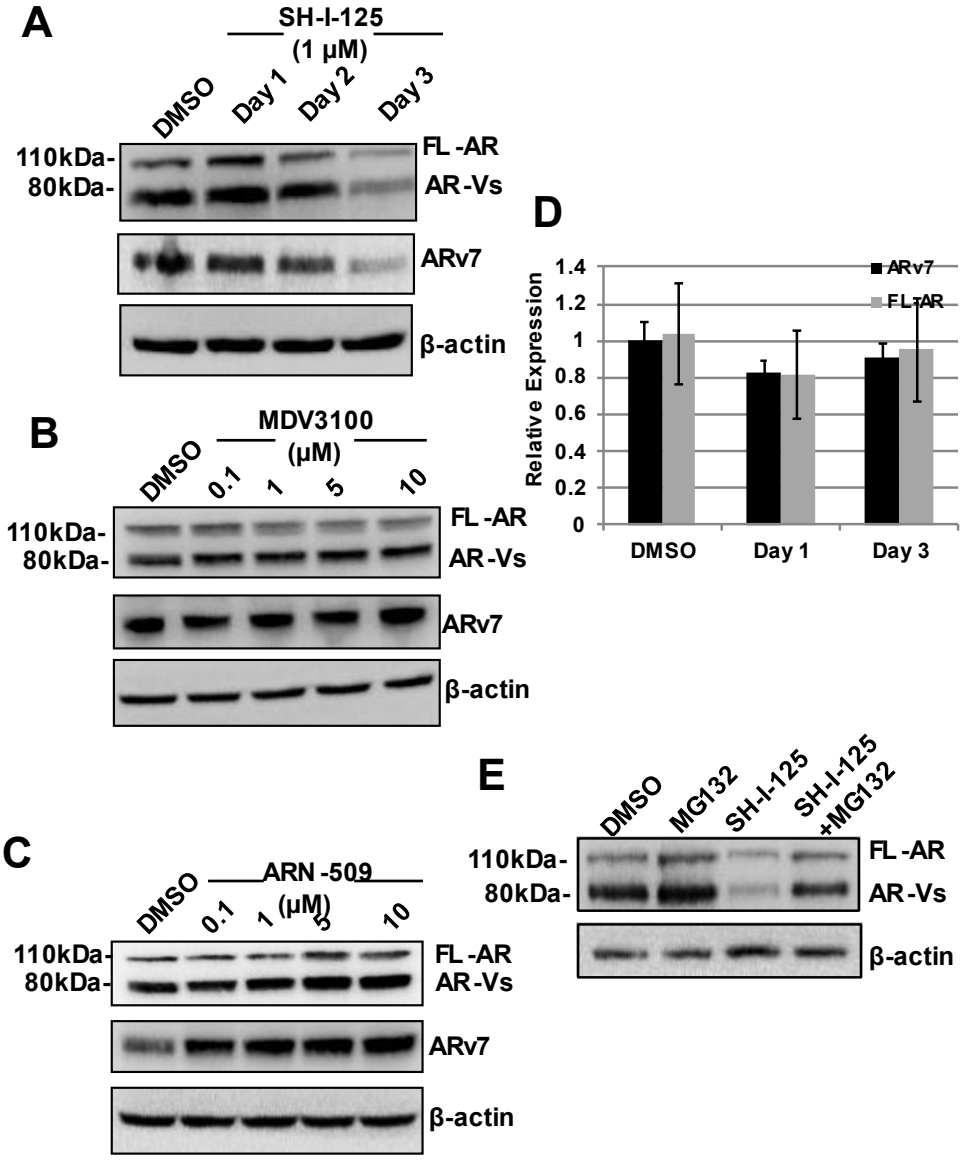


Figure 5: SH-I-125 decreases cellular levels of AR and AR-V7 in prostate cancer cells by proteasomal degradation
22Rv1 cells were treated with (A) 1 μM SH-I-125 for 1-3 days or with the indicated doses of (B) MDV3100 or (C) ARN509 for a period of 3 days. AR and AR-V7 levels were assessed by Western blotting. β-actin was used as a loading control. (D) 22Rv1 cells were treated with SH-I-125 (1 μM) for 24 and 72 hrs. AR and AR-V7 expression levels were assessed by qRT-PCR and normalized to GAPDH expression levels. (E) 22Rv1 cells were treated with SH-I-125 (1 μM) for a period of 3 days. MG132 (5 μM) was added on Day 2. AR expression was assessed by Western blotting. β-actin was used as a loading control.

3.8. SH-I-125 prevents the growth of prostate cancer cells that are resistant to clinical anti-androgens

The emergence of resistance to clinical anti-androgens is an ongoing problem in the treatment of CRPC. To establish whether SH-I-125 can prevent the growth of drug-resistant prostate cancer cells, we generated VCaP- and 22Rv1-MDV3100-resistant cells (VCaP-MDVR and 22Rv1-MDVR) by prolonged culturing of these cell lines in the

presence of MDV3100 (details described under Materials and Methods). VCaP-MDVR cells, but not 22Rv1-MDVR cells, showed increased expression of AR splice variants compared to control (Fig. 6A and 6B, upper pannels). Both VCaP and 22Rv1 cells acquired resistance to second generation anti-androgens, evidenced by their continued proliferation in the presence of MDV3100 and ARN509 (Fig. 6A and 6B, lower panels). However, treatment of VCaP-MDVR and 22Rv1-MDVR cells with SH-I-125 showed that it was effective in preventing the growth and inducing caspase activation in both the drug-resistant cell lines (Fig. 6C and 6D).

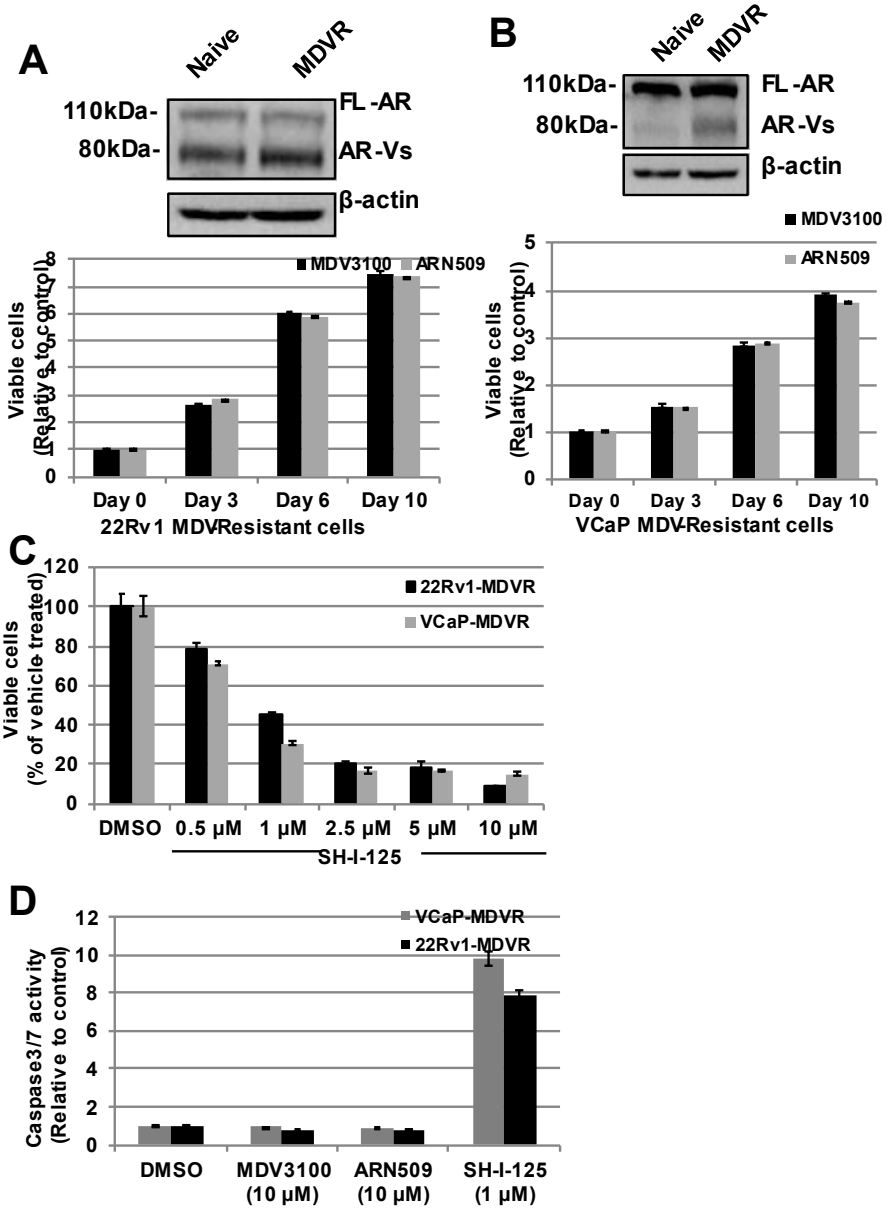


Figure 6: SH-I-125 prevents growth and induces apoptosis in MDV3100-resistant prostate cancer cells
(A and B; upper panels) AR expression was evaluated in 22Rv1-MDVR and VcaP-MDVR cells by Western blot. (A and B; lower panels) 22Rv1-MDVR and VCaP-MDVR cells were treated with MDV3100 and ARN509 (10 μM) for the indicated period of time. (C) 22Rv1-MDVR and VCaP-MDVR cells were treated with the indicated concentrations of SH-I-125 for 3 days. Cell viability was measured using the MTT assay. Data represents mean of three independent experiments with quadruplicate samples; bars, SEM. (D) VCaP-MDVR and 22Rv1-MDVR were treated with MDV3100, ARN509 (10 μM) and SH-I-125 (1 μM) for 3 days. Caspase 3/7 activity was measured using Caspase-Glo 3/7 substrates. Data represents mean of three independent experiments with quadruplicate samples; bars, SEM.

3.9. Drug-resistant cells retain AR activity in the presence of second-generation anti-androgens, but not upon treatment with SH-I-125

VCaP-MDVR and 22Rv1-MDVR stably expressing ARE-luciferase construct (VCaP-MDVR-ARE-luc and 22Rv1-MDVR-ARE-luc) were generated as described in the materials and methods section. Luciferase activity, a measure of AR activation, was only reduced by 50% in VCaP-MDVR-ARE-luc cells by MDV3100 and ARN509 treatment, which is in contrast to an approximate 80% reduction in VCaP-ARE-luc cells (Fig. 7A and Fig. 2A). Similarly, in 22Rv1-MDVR-ARE-luc cells, luciferase activity remained unchanged from control upon treatment with a range of doses of MDV3100 and ARN509, which is in contrast to the decrease in luciferase activity seen in 22Rv1-ARE-luc cells treated in the same manner (Fig. 7B and Fig. 3A). However, SH-I-125 retained its inhibitory effect on AR activity in both drug-resistant cell lines.

Furthermore, while MDV3100 continued to inhibit PSA expression in VCaP-MDVR cells, AR expression was not attenuated as seen before in naïve-VCaP cells (Fig. 7C, Fig. 7D and Fig. 2B), suggesting that although AR activity is partially reduced, evidenced by decreased PSA expression, its continued expression indicates that it could be involved in mediating alternative transcriptional programs. SH-I-125 decreased AR nuclear localization and PSA expression in VCaP-MDVR cells in a manner similar to that seen in naïve-VCaP cells (Fig. 7C, Fig. 7D and Fig. 2B).

Although AR nuclear localization remained unaffected by MDV3100 and ARN509 treatment, SH-I-125 treatment decreased AR nuclear levels and caused it to translocate to the cytoplasm in 22Rv1-MDVR cells (Fig. 7E and Fig. 7F), suggesting SH-I-125 has the ability to decrease nuclear localization of AR and its splice variants in androgen-independent and anti-androgen-resistant prostate cancer cells.

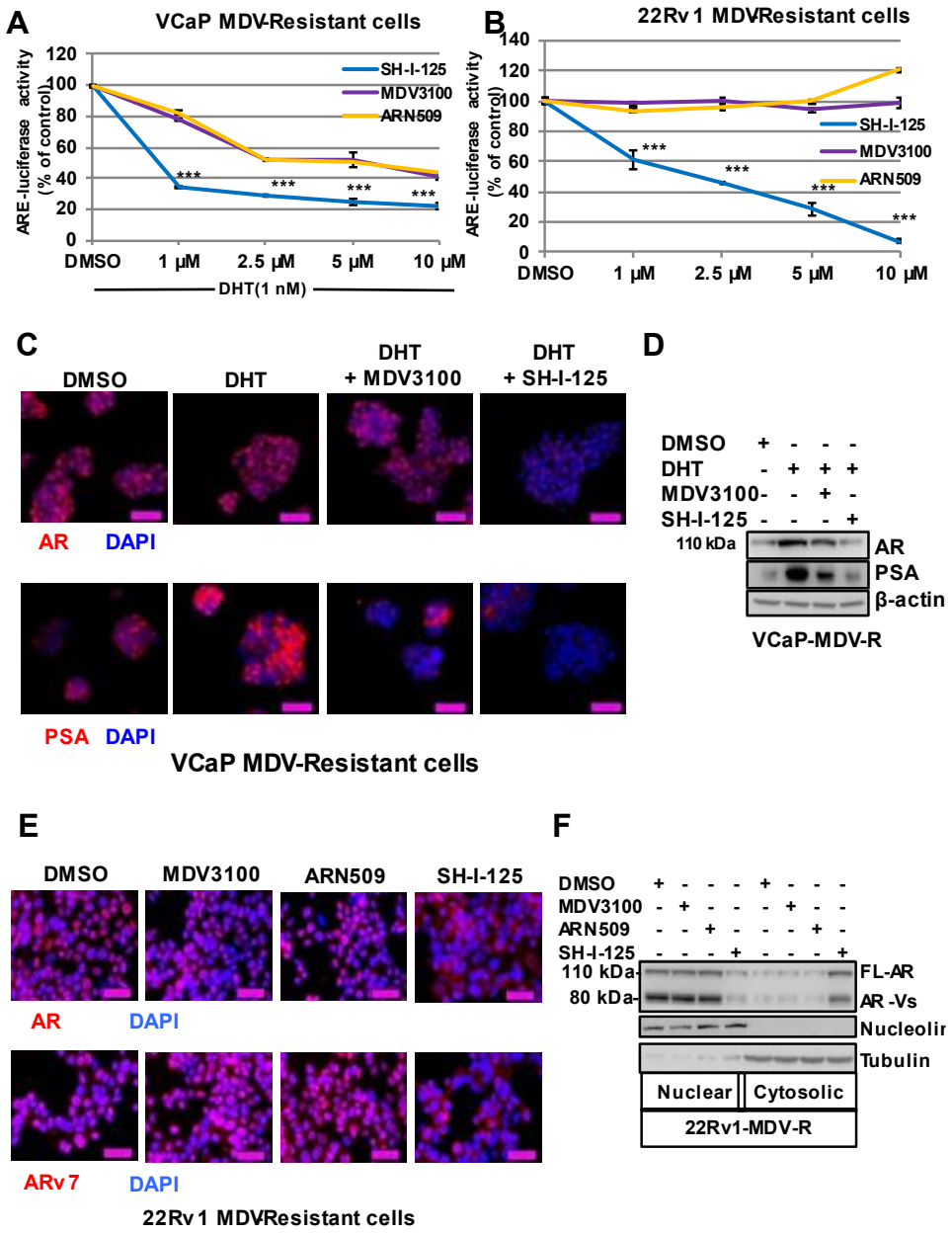


Figure 7: SH-I-125 retains its ability to prevent AR transactivation in MDV3100-resistant prostate cancer cells
(A) VCaP-MDVR-ARE-luc cells and (B) 22Rv1-MDVR-ARE-luc cells were treated with a range of doses of MDV3100, ARN509 and SH-I-125 for 24 hrs. Luciferase activity was measured and plotted as a percent of control. Data represents mean of three independent experiments with quadruplicate samples; bars, SEM. ***, p value <0.001 determined by ANOVA. VCaP-MDVR cells were treated with DHT (1nM) in the absence and presence of MDV3100 (10 μM) or SH-I-125 (1 μM) for 24 hrs. AR and PSA expression levels were assessed by (C) immunofluorescence staining and (D) Western blotting. 22Rv1-MDVR cells were treated with MDV3100 (10 μM), ARN509 (10 μM) or SH-I-125 (1 μM) for 24 hrs. AR and AR-V7 expression was assessed by (E) immunofluorescence staining and (F) Western blotting of nuclear-cytoplasmic cell lysate fractions. Tubulin and nucleolin were used as a cytoplasmic and nuclear fraction loading controls, respectively.

4. Discussion

The successful inhibition of AR signaling is clinically sought-after in the therapeutic management of castration-resistant prostate cancer. Several different approaches have attempted to achieve this goal, including surgical

castration,[20] hormonal analogues and antagonists,[21] inhibitors of steroidogenic enzymes[22] and competitive inhibitors of the AR such as second-generation anti-androgens, MDV3100 and ARN509 [10, 23]. Although all of these approaches have demonstrated varying degrees of success in the clinic, their long-term benefits are questionable because of the emergence of resistance and eventual progression of the cancer. [14] The continued activation of AR signaling in spite of castrate conditions and the expression of AR splice variants contribute to mediating the aggressive phenotype of castration-resistant prostate cancer. Therapies which disrupt AR signaling and growth in castration-resistant and drug-resistant prostate cancer are required for the effective treatment of patients with advanced disease.

Although mahanine effectively suppresses prostate cancer cell growth and disrupts AR signaling, being a natural product, its extraction and purification for clinical purposes is cumbersome, yields low amounts of purified product and batch-to-batch variation in biological activity. In spite of the challenges associated with development of synthetic analogues of natural compounds, including the presence of complex chiral centers and the potential loss of activity due to structural modifications, these compounds have demonstrated tremendous therapeutic benefit in multiple disease types. To overcome the shortcomings of the use of natural compounds in the clinic, we synthesized and screened a series of synthetic carbazole compounds and identified one lead compound SH-I-125, which could disrupt AR signaling and inhibit prostate cancer cell growth more effectively than second generation anti-androgens, MDV3100 and ARN509, and naturally-dervived mahanine.

To obtain lead compound SH-I-125, three structural modifications have been made to mahanine: (1) the phenol group was transformed into methoxyl group; (2) the position of the substituted methyl group was changed; the 2*H* chromene was replaced with an aromatic sulfonate. Since SH-I-125 was significantly more potent than mahanine in disrupting AR signaling and inhibiting the growth of castration-resistant prostate cancer cells, a question that remains to be addressed is which chemical modification caused the change in potency. We are currently looking into the structure-activity relationships of this novel carbazole which will reveal specific moieties that are essential for enhanced activity in terms of inhibiting AR transactivation and prevent prostate cancer cell growth.

Interestingly, SH-I-125 demonstrated differential effects on growth inhibition of tumorigenic vs non-tumorigenic cells. SH-I-125 does not decrease the viability or induce cell death in non-tumorigenic, primary prostatic epithelial cells (PrEC) as seen in tumorigenic 22Rv1 cells treated with SH-I-125. This suggests that SH-I-125 could be targeting pathways which are up-regulated in cancer cells and facilitate the tumorigenic phenotype of increased growth and altered metabolism. While cancer cells are known to be addicted to survival pathways such as AKT and MAPK signalling which promote proliferation and inhibit apoptosis, non-tumorigenic cells do not exhibit this dependency. We have found that SH-I-125 decreases the levels of p-AKT in prostate cancer cells (unpublished data), thereby indicating its ability to target a pathway known to be up-regulated in cancer vs normal cells. However, additional studies will be needed to further compare the effects of these agents on tumorigenic vs non-tumorigenic cell types and thereby explain why the former is more sensitive.

In androgen-dependent cells, SH-I-125 demonstrated comparable potency to MDV3100 and ARN509 in the inhibition of ligand-dependent AR. In androgen-independent cells, while SH-I-125 retains its ability to inhibit AR signaling, MDV3100 and ARN509 are notably less effective, and are unable to inhibit AR activity to the same extent as in the case of androgen-dependent cells. This suggests that although second generation anti-androgens are highly effective in competitively inhibiting ligand-dependent AR activation, their antagonistic effects on AR signaling are compromised in cells which express AR splice variants and are castration-resistant. This data raises concerns about the long-term effectiveness of these therapies in advanced prostate cancer which has progressed beyond androgen dependence and relies on alternative means of AR activation and the expression of AR splice variants. Importantly, SH-I-125 not only inhibits AR signaling in androgen-dependent and –independent scenarios, it demonstrates efficacy against the activation of the AR-NTD by growth factors in a manner similar to mahanine. AR-NTD inhibition is indicative of the ability to thwart growth factor mediated transactivation of AR and its splice variants, which lack the AR LBD but retain the AR NTD. This suggests that SH-I-125 could effectively disrupt AR signaling irrespective of its mode of activation. This is of significance in prostate cancer, where each tumor comprises a heterogeneous population of cells capable of paracrine and autocrine production of growth factors which can activate signaling cascades converging upon AR activation.

Although it has been reported that second generation anti-androgens induce caspase activation and apoptosis in androgen-dependent

cells, our studies showed that these events were not induced in VCaP cells treated with 10uM of MDV3100 or ARN509. This discrepancy in findings could be attributed to differences in culture conditions and passage number of the cells. However, SH-I-125 induced caspase activation and apoptosis in androgen-dependent and –independent prostate cancer cell lines, suggesting that it is more effective than clinical anti-androgens in inducing apoptotic cell death in prostate cancer. It remains to be determined whether caspase-3 and -7 activation is solely responsible for mediating cell death induced by SH-I-125, or whether other signaling pathways are involved.

Emergence of drug resistance is an ongoing problem in castration-resistant prostate cancer therapy. Several mechanisms have been explored to explain MDV3100 resistance, including the expression of AR splice variants, mutations in the AR LBD rendering it unresponsive to MDV3100 and parallel activation of signaling pathways which promote proliferation and invasive phenotypes. We developed two MDV3100-resistant (MDVR) cell lines by prolonged culturing of VCaP and 22Rv1 cells in 10 μM MDV3100. These MDVR cells continued to proliferate in the presence of 10 μM MDV3100, and were resistant to the inhibitory effects of MDV3100 and ARN509 on AR activation. Interestingly, SH-I-125 effectively inhibited the growth, disrupted AR activation, and induced cell death in MDVR cells, suggesting that SH-I-125 is effective against drug-resistant prostate cancer cells. It is worth noting that SH-I-125 induced caspase activity by 8-10 fold in VCaP-MDVR and 22Rv1-MDVR cells, respectively, while caspase induction was only 3-6 fold in the corresponding naïve VCaP and 22Rv1 cell lines, suggesting that drug-resistant cells were more susceptible to apoptosis induction by SH-I-125. However, further studies are needed to assess mechanisms by which the MDVR cell lines have developed resistance to clinical anti-androgens, and whether SH-I-125 reverses the events that result in the resistant phenotype. Although SH-I-125 was found to be effective in disrupting AR transactivity, preventing growth and inducing apoptosis in prostate cancer cells, but not non-tumorigenic prostatic epithelial cells, its *in vivo* effects and potential side effects are yet to be determined in castration-resisant and anti-androgen resistant xenograft models. Past several years, AR degrading PROTAC's are also evolving as potential AR degraders and might have significant roles in regulating AR signaling in AR dependent and CRPC [24-27]. Although, it is too early to predict which of those AR PTOTACs will eventually will be well tolerated and approved for the treatment of CRPC patient.

5. Conclusions

The data presented herein indicates that SH-I-125, a synthetic novel carbazole, demonstrates improved ability over second-generation anti-androgens MDV3100 and ARN509 in the inhibition of AR signaling and growth in castration-resistant cells and drug-resistant prostate cancer cells, thereby highlighting its therapeutic potential in patients with castration-resistant prostate cancer where the AR plays a central role in tumor growth and progression.

Acknowledgements

The authors thank Dr. Satoru Kyo of Kanazawa University for the hTERT promoter luciferase reporter construct, Dr. Linda Penn of Ontario Cancer Institute for the c-myc promoter luciferase reporter construct, Dr. Marianne Sadar of University of British Columbia for AR-NTD-Gal4DBD and Gal4UAS-TATA-luciferase vectors, and Dr. Winship Herr of Université de Lausanne for VP16-Gal4DBD vector. The authors acknowledge the use of the Genomics and Epigenomics Shared Resource and the Tissue Culture Shared Resource in Lombardi Comprehensive Cancer Center for various studies.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest.

Funding

This work was supported by National Institutes of Health R01 CA131123 to Dr. P. P. Banerjee.

Grant Support: NIH R01CA131123 to Dr. P. P. Banerjee.

Running Title: SH-I-125 overcomes anti-androgen resistance in prostate cancer cells

Conflict of interest: All authors declare no conflict of interest

Author's Contributions

KSA is responsible for the experimental design, execution, data interpretation, data analyses and writing of the manuscript. SH is responsible for the design, synthesis, purification and characterization of SH-I-125, carbazole analogs and synthetic mahanine as well editing of the manuscript. JBC performed several Western blots that are included in the manuscript; MB provided oversight for the synthesis of the chemical library including SH-I-125 and synthetic mahanine. PB directed the experimental design and overall supervision, the establishment of ARE-Luciferase cells, the development of MDV-resistant cells and edited the manuscript. All authors have read and approved the final manuscript.

References

1. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2022. *CA Cancer J Clin* 2022, 72:7-33.
2. Feldman BJ, Feldman D: The development of androgen-independent prostate cancer. *Nat Rev Cancer* 2001, 1:34-45.
3. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T: Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res* 2001, 61:3550-3555.
4. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C: From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc Natl Acad Sci U S A* 1999, 96:5458-5463.
5. Wen Y, Hu MC, Makino K, Spohn B, Bartholomeusz G, Yan DH, Hung MC: HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res* 2000, 60:6841-6845.
6. Tilley WD, Buchanan G, Hickey TE, Bentel JM: Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. *Clin Cancer Res* 1996, 2:277-285.
7. Ferraldeschi R, Sharifi N, Auchus RJ, Attard G: Molecular pathways: Inhibiting steroid biosynthesis in prostate cancer. *Clin Cancer Res* 2013, 19:3353-3359.
8. Sun S, Sprenger CC, Vessella RL, Haugk K, Soriano K, Mostaghel EA, Page ST, Coleman IM, Nguyen HM, Sun H, et al: Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *J Clin Invest* 2010, 120:2715-2730.
9. Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, Chen H, Kong X, Melamed J, Tepper CG, et al: A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. *Cancer Res* 2009, 69:2305-2313.
10. Clegg NJ, Wongvipat J, Joseph JD, Tran C, Ouk S, Dilhas A, Chen Y, Grillot K, Bischoff ED, Cai L, et al: ARN-509: a novel antiandrogen for prostate cancer treatment. *Cancer Res* 2012, 72:1494-1503.
11. Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, de Wit R, Mulders P, Chi KN, Shore ND, et al: Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med* 2012, 367:1187-1197.
12. Beer TM, Armstrong AJ, Rathkopf DE, Loriot Y, Sternberg CN, Higano CS, Iversen P, Bhattacharya S, Carles J, Chowdhury S, et al: Enzalutamide in Metastatic Prostate Cancer before Chemotherapy. *N Engl J Med* 2014.
13. Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KA, Dehm SM: Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines. *Cancer Res* 2012, 73:483-489.
14. Joseph JD, Lu N, Qian J, Sensintaffar J, Shao G, Brigham D, Moon M, Maneval EC, Chen I, Darimont B, Hager JH: A clinically relevant androgen receptor mutation confers resistance to second-generation antiandrogens enzalutamide and ARN-509. *Cancer Discov* 2013, 3:1020-1029.
15. Korpal M, Korn JM, Gao X, Rakiec DP, Ruddy DA, Doshi S, Yuan J, Kovats SG, Kim S, Cooke VG, et al: An F876L mutation in androgen receptor confers genetic and phenotypic resistance to MDV3100 (enzalutamide). *Cancer Discov* 2013, 3:1030-1043.
16. Arora VK, Schenkein E, Murali R, Subudhi SK, Wongvipat J, Balbas MD, Shah N, Cai L, Efstathiou E, Logothetis C, et al: Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade. *Cell* 2013, 155:1309-1322.
17. Amin KS, Jagadeesh S, Baishya G, Rao PG, Barua NC, Bhattacharya S, Banerjee PP: A naturally derived small molecule disrupts ligand-dependent and ligand-independent androgen receptor signaling in human prostate cancer cells. *Mol Cancer Ther* 2014, 13:341-352.
18. Nazareth LV, Weigel NL: Activation of the human androgen receptor through a protein kinase A signaling pathway. *J Biol Chem* 1996, 271:19900-19907.
19. Chen T, Wang LH, Farrar WL: Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells. *Cancer Res* 2000, 60:2132-2135.
20. Huggins C, Stephens, R. C. & Hodges, C. V: The effects of castration on
advanced carcinoma of the prostate gland. *Arch Surg* 1941, 43:209.
21. Labrie F, Belanger A, Luu-The V, Labrie C, Simard J, Cusan L, Gomez J, Candas B:

Gonadotropin-releasing hormone agonists in the treatment of prostate cancer. *Endocr Rev* 2005, 26:361-379.

22. de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman OB, Jr., Saad F, et al: Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011, 364:1995-2005.

23. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, et al: Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 2009, 324:787-790.

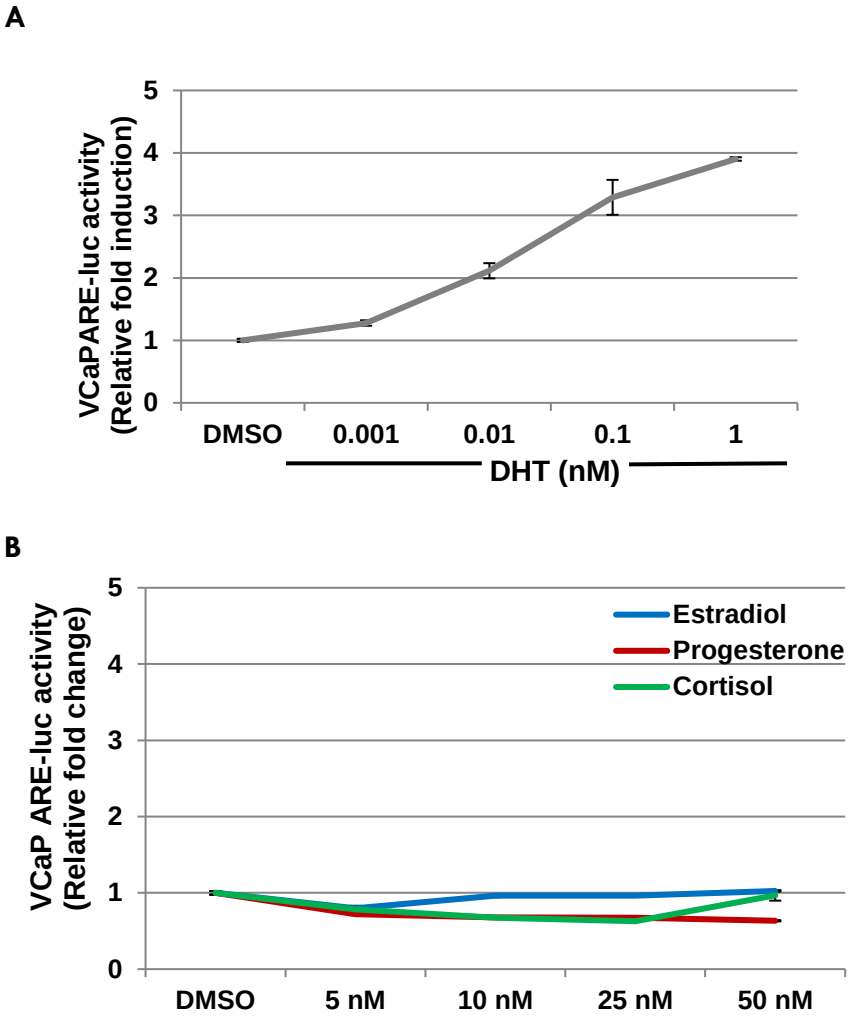
24. Salami J, Alabi S, Willard RR, Vitale NJ, Wang J, et al: Androgen receptor degradation by the proteolysis-targeting chimera ARCC-4 outperforms enzalutamide in cellular models of prostate cancer drug resistance. Commun Biol. 2018;1:100.

25. Han X, Wang C, Qin C, Xiang W, Fernandez-Salas E, Yang CY, et al: Discovery of ARD-69 as a Highly Potent Proteolysis Targeting Chimera (PROTAC) Degrader of Androgen Receptor (AR) for the Treatment of Prostate Cancer. J Med Chem. 2019 Jan 24;62(2):941-964.

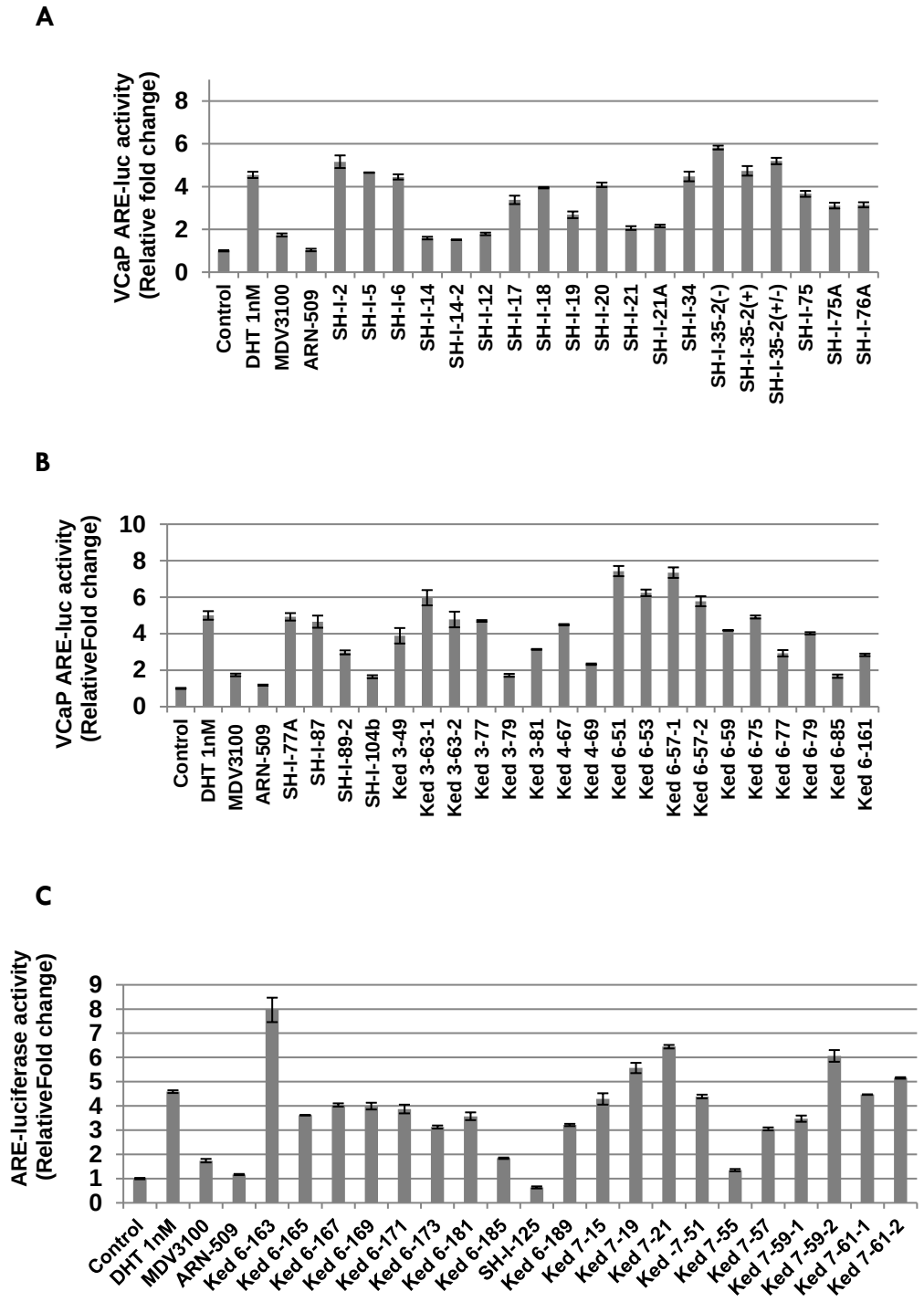
26. Chen L, Han L, Mao S, Xu P, Xu X, Zhao R, et al: Discovery of A031 as effective proteolysis targeting chimera (PROTAC) androgen receptor (AR) degrader for the treatment of prostate cancer. Eur J Med Chem. 2021, 216: 113370.

27. Xiang W, Zhao L, Han X, Qin C, Miao B, et al: Discovery of ARD-2585 as an Exceptionally Potent and Orally Active PROTAC Degrader of Androgen Receptor for the Treatment of Advanced Prostate Cancer. J Med Chem. 2021 Sep 23;64(18):13487-13509.

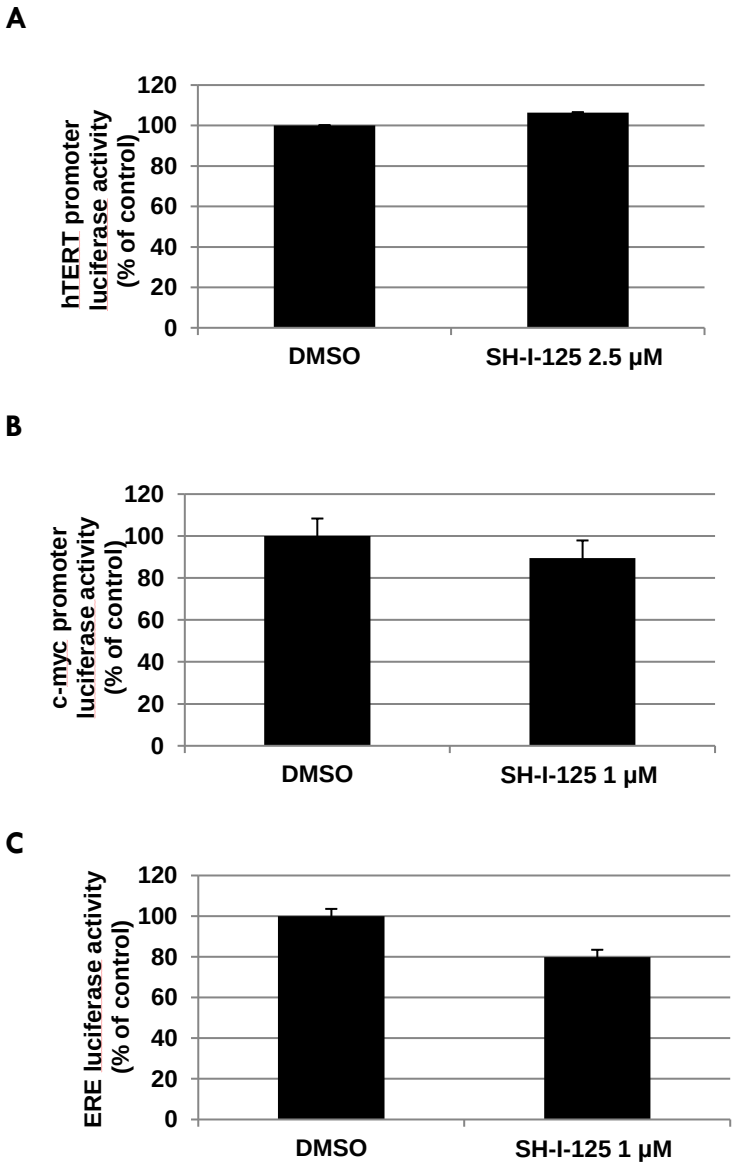
SUPPLEMENTARY MATERIAL



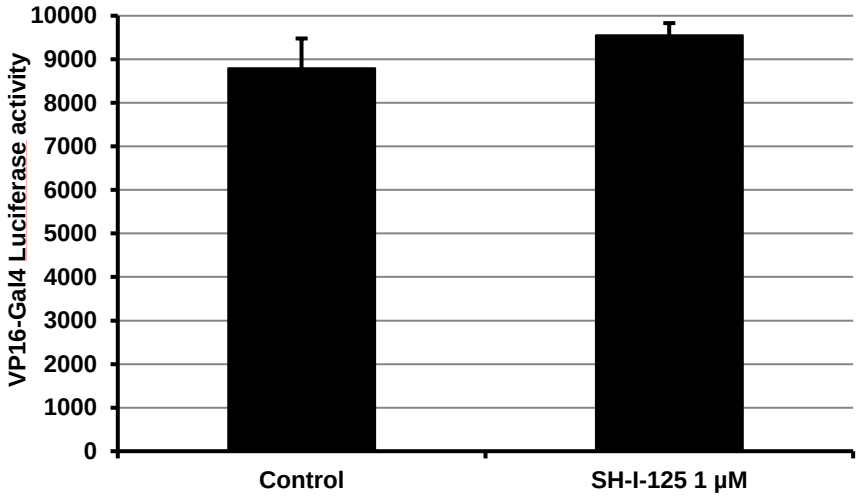
Supplementary Figure 1. Dose-response of DHT, Estradiol, Progesterone and Cortisol on VCaP-ARE-luc cells. (A) Dose-response curve for luciferase activity induction upon treatment with DHT for 24 hrs. (B) VCaP-ARE-luc cells were treated with indicated concentrations of estradiol, progesterone and cortisol for 24 hrs. Luciferase activity was measured as a percent of control. Columns, mean of three independent experiments with quadruplicate samples; bars, SEM.



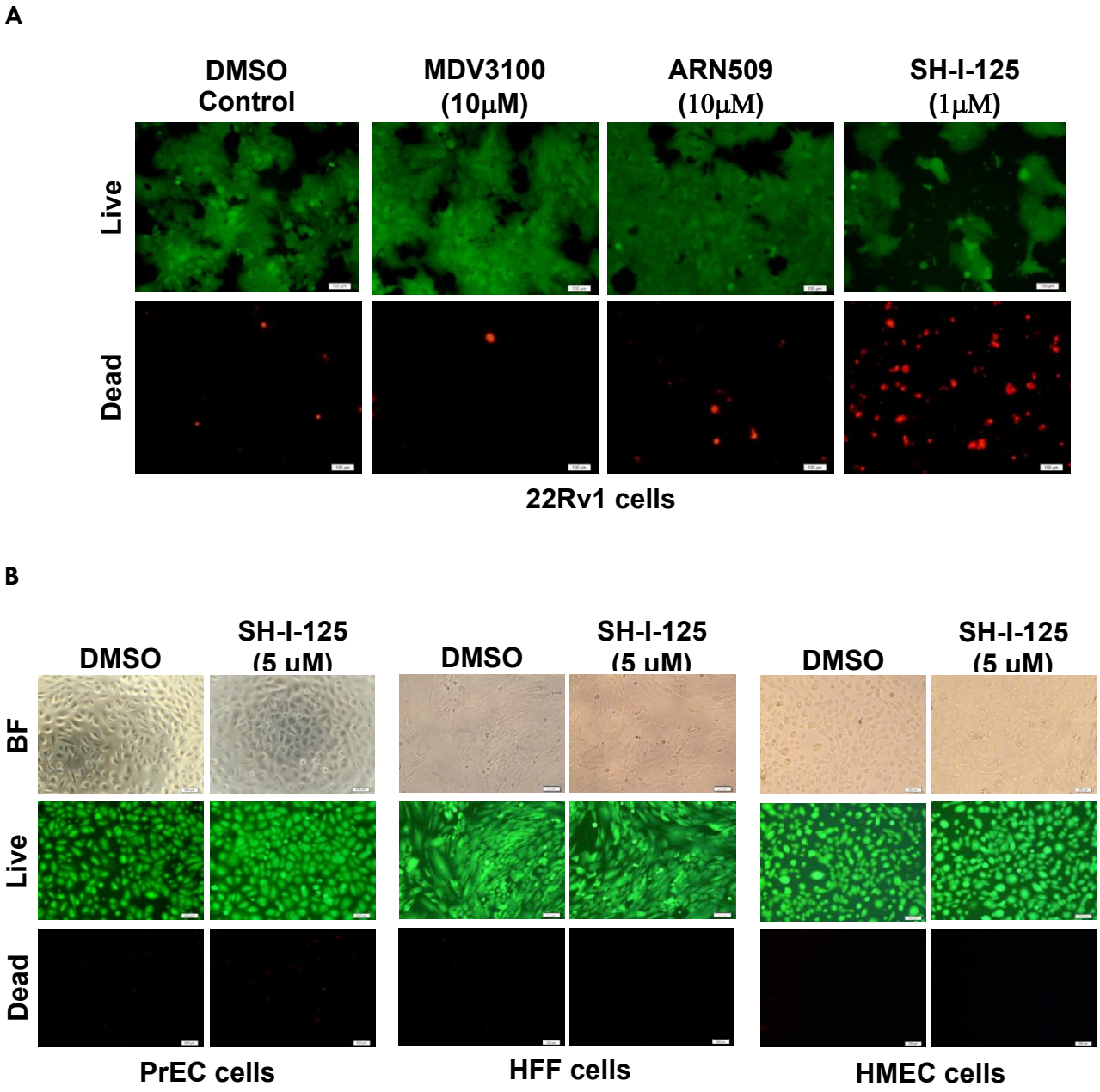
Supplementary Figure 2. Screening of library of synthetic carbazoles using VCaP-ARE-luc cells. (A), (B) and (C) VCaP-ARE-luc cells were treated with MDV3100 (5 μM), ARN-509 (5 μM) and a library of synthetic carbazoles (5 μM) in the presence of DHT (1nM) for 24 hrs. Luciferase activity was determined relative to the control. Columns, mean of three independent experiments with quadruplicate samples; bars, SEM.



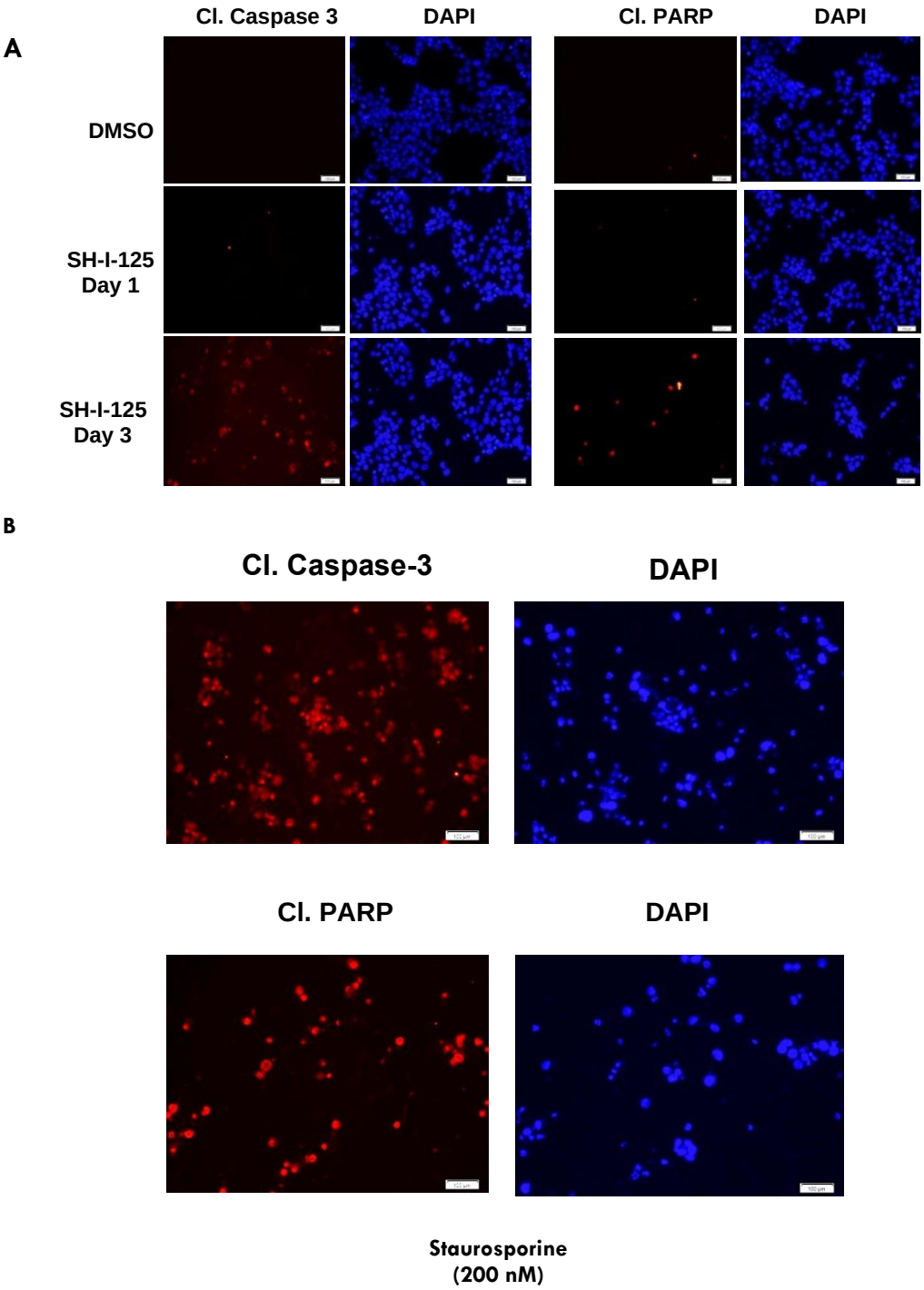
Supplementary Figure 3. Effect of SH-I-125 on hTERT and c-myc promoter luciferase activity. 22Rv1 cells were transfected with (A) hTERT promoter-luciferase reporter construct, (B) c-myc promoter-luciferase reporter construct or (C) ERE luciferase reporter construct and Renilla luciferase plasmids (pRL-TK-Luc) and treated with the indicated doses of SH-I-125 for 24 hrs. Promoter activity was determined after normalization with Renilla luciferase activity. Columns, mean of three independent experiments with quadruplicate samples; bars, SEM.



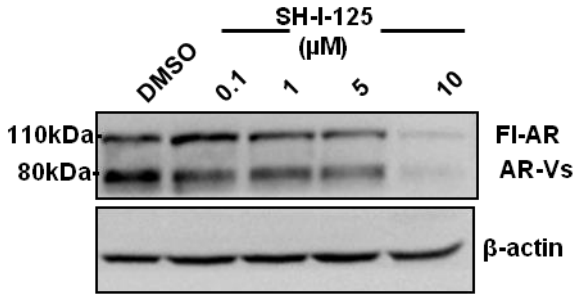
Supplementary Figure 4. Effect of SH-I-125 on VP16-Gal4DBD activity. 22Rv1 cells were co-transfected with VP16 activation domain-Gal4DBD fusion protein and Gal4UAS-TATA-luciferase reporter and treated with SH-I-125 for 24 hours. Luciferase activity was measured and normalized with R. luciferase. Columns, mean of three independent experiments with quadruplicate samples; bars, SEM.



Supplementary Figure 5. Effect of SH-I-125 on cell death in tumorigenic vs non-tumorigenic cells. (A) 22Rv1 cells were treated with MDV3100, ARN-509 and SH-I-125 (1 μM) as indicated for 3 days. The live cells stained with calcein AM appear green and the dead cells stained with EthD-III appear red. (B) primary prostate epithelial cells (PrEC), primary human foreskin fibroblasts (HFF) and primary human mammary epithelial cells (HMEC) cells were treated with SH-I-125 (5 μM) for 3 days and stained as described in (A). (C) 22Rv1 cells were treated with staurosporine for 4 hrs and live and dead cell staining was performed as in (A).



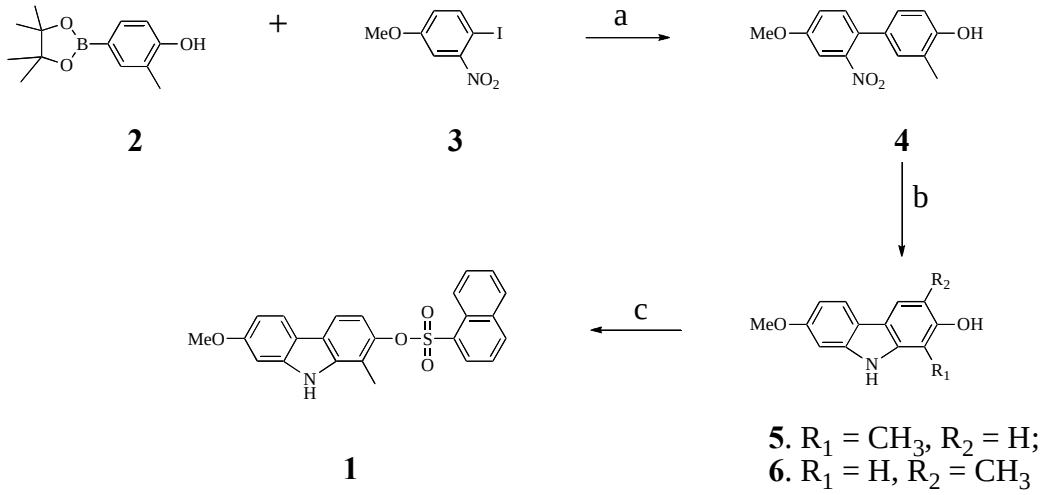
Supplementary Figure 6. Effect of SH-I-125 or staurosporine on apoptotic markers in 22Rv1 cells. 22Rv1 cells were treated with (A) SH-I-125 (1 μM) for three days or (B) staurosporine (200 nM) for four hours and the presence of cleaved caspase-3 and cleaved PARP was determined by immunofluorescence staining.



Supplementary Figure 7. Effect of SH-I-125 on AR levels in 22Rv1 cells. 22Rv1 cells were treated with the indicated doses of SH-I-125 for 3 days. AR levels were assessed by Western blotting. β-actin was used as a loading control.

SUPPLEMENTARY METHODS

Synthesis of SH-I-125



Scheme 1. Synthesis of SH-I-125 [1]

Reagents and conditions: (a). $Pd(PPh_3)_4$, K_2CO_3, Toluene-ethanol-water, reflux, 87%; (b). PPh_3, 1,3–DCB, 250°C, 200 W, microwaves; 42% for **5** and 47% for **6**; (c). 1-naphthalenesulfonyl chloride, DCM-TEA, 85%.

The Palladium-catalyzed cross-coupling reaction was carried out between the commercially available compounds 2-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenol (**2**) and 1-iodo-4-methoxy-2-nitrobenzene **(3)** providing the biphenyl-4-ol compound (**4**) in a yield of 87%. Although the one-step cyclization of a 2-nitrobiphenyl system was previously reported using different conditions[1-3], it was confirmed that those conditions are not suitable to build the carbazole ring from **4** because of the free phenol group. Several conditions were investigated to introduce the carbazole ring from **4**, the satisfied yield was achieved until the reaction was optimized under the microwave condition yielding the two known regioisomeric carbazoles **5** (42%) and **6** (47%) in a form of mixture as expected[4]. Despite the steric hindrance expected in the cyclization to compound **5**, compounds **5** and **6** were produced in an approximately 1:1 ratio (TLC and NMR). Varying the solvent (1,2- or 1,3-dichlorobenzene, DMF, xylenes), the equivalents of triphenylphosphine as well as the temperature and the power of the microwave resulted in only minimal changes to the ratio of regioisomers. Treated with 1-naphthalenesulfonyl chloride in the presence of triethyalamine, **5** was smoothly transformed into the target sulfonic ester SH-I-125 **(1)** with a yield of 85%.

All reagents and solvents were purchased from commercial suppliers and used as received unless noted otherwise. Flash column chromatography separations were done on a Biotage SP1 system monitoring at 254 and 310 nm. NMR spectra were recorded on a Varian 400 spectrometer at 25 °C, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. The chemical shifts are expressed in ppm downfield from TMS as an internal standard. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 glass slides. The structure of the synthesized compounds follows unequivocally from the mode of synthesis and the *m/z* values found in their low- and high-resolution mass spectra, TLC and NMR spectroscopy verified the purity.

4'-methoxy-3-methyl-2'-nitrobiphenyl-4-ol (4). 4-Hydroxyphenylboronic acid (**2**) (2.3 g, 10 mmol) was added to a solution of 4-iodo-3-nitroanisole (**3**) (3.1 g, 11 mmol) in toluene-ethanol-water (30 ml, v/v/v=4/2/1), followed by $Pd(PPh_3)_4$ (350 mg, 0.3 mmol) and K_2CO_3 (5.3 g, 50 mmol), the mixture was refluxed for 6 h. After cooling to room temperature, the mixture was filtered through a celite pad. The filtrate was concentrated, diluted with DCM (100 ml) and washed with saturated brine (3 × 30 ml). After concentration and chromatography (hexane/acetone = 6/1) afforded 4'-methoxy-3-methyl-2'-nitrobiphenyl-4-ol (**4**) (2.3 g, 87%). ^{1}H NMR (400 MHz, $CDCl_3$), δ 7.31 (m, 2 H), 7.12 (dd, J = 2.8 Hz, 8.4 Hz, 1 H), 7.04 (s, 1 H), 6.98 (d, J = 8.0 Hz, 1 H), 6.78 (d, J = 8.0 Hz, 1 H), 3.88 (s, 3 H), 2.26 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$), δ 158.7, 153.7, 132.7, 130.6, 129.5, 128.3, 126.7, 124.1, 118.5, 115.1, 108.8, 55.8, 15.7; MS (TOF-ES$^+$): 260.3 *m/z* $[M+1]^+$

7-methoxy-1-methyl-9*H*-carbazol-2-ol (**5**) and **7-methoxy-3-methyl-9*H*-carbazol-2-ol** (**6**)
A mixture of **4** (260 mg, 1 mmol), triphenylphosphine (1.04 g, 4.0 mmol) and 1,3-dichlorobenzene (3 ml) was heated in a microwave (200 °C, powermax) for 6 h. After concentration under reduced pressure and chromatography (tolunen/acetone = 3/1) afforded the known compounds **5** (94 mg, 42%) and **6** (107 mg, 47%). NMR data were identical with those of reported [4].

7-methoxy-1-methyl-9*H*-carbazol-2-yl naphthalene-1-sulfonate (SH-I-125).
1-naphthalenesulfonyl chloride (136 mg, 0.6 mmol) was added to a solution of **5** (90 mg, 0.4 mmol) in anhydrous dichloromethane (2 ml) in the presence of Et_3N (0.5 ml) under N_2 with stirring at 0 °C. After stirring for 2 h, the mixture was concentrated and chromatographed (hexane/acetone = 2/1) to give compound **1** (141 mg, 85%) as with solid. ^{1}H NMR (400 MHz, $CDCl_3$), δ 8.82 (d, J = 8.4 Hz, 1 H), 8.06 (d, J = 8.4 Hz, 1 H), 8.04 (d, J = 7.6 Hz, 1 H), 7.91 (d, J = 8.4 Hz, 1 H), 7.67 (m, 2 H), 7.58 (t, J = 8.0 Hz, 1H), 7.39 (t, J = 8.0 Hz, 1 H), 7.35 (d, J = 8.4 Hz, 1 H), 6.80 (d, J = 2.0 Hz, 1H), 6.70 (dd, J = 2.0 Hz, J = 8.4 Hz, 1 H), 6.25 (d, J = 8.4 Hz, 1 H), 3.76 (s, 3 H), 2.28 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$), δ 159.06, 145.29, 141.38, 139.35, 135.53, 134.16, 131.90, 130.92, 128.93, 128.91, 128.67, 127.31, 125.30, 124.00, 121.63, 121.03, 116.98, 116.80, 113.83, 113.48, 1087.72, 94.84, 55.56, 11.23; HRMS (TOF-ES$^+$): *m/z* $[M+1]^+$ calcd for $C_{24}H_{19}NO_4S$: 418.1032; found 418.1029.

Reference:
1. Freeman AW, Urvoy M, Criswell ME. Triphenylphosphine-mediated reductive cyclization of 2-nitrobiphenyls: a practical and convenient synthesis of carbazoles. J Org Chem. 2005;70:5014-9.
2. Appukkuttan P VdEE, Dehaen W. Microwave-enhanced cadogan cyclization: an easy access to the 2-substituted carbazoles and other fused heterocyclic systems. Synlett. 2005;1:127-33.
3. Sanz R EJ, Pedrosa MR, Aguado R, Arnáiz F J. Dioxomolybdenum(VI)-catalyzed reductive cyclization of nitroaromatics. synthesis of carbazoles and indoles. Adv Synth Catal. 2007;349:713-8.
4. Sheikh KD, Banerjee PP, Jagadeesh S, Grindrod SC, Zhang L, Paige M, et al. Fluorescent epigenetic small molecule induces expression of the tumor suppressor ras-association domain family 1A and inhibits human prostate xenograft. J Med Chem. 2010;53:2376-82.