Direct blockade of AR binding to its target genes for the treatment of advanced prostate cancer by a novel anti-androgen SBF-1

Ahmed Elgehama[1], Wang BINGLIN[1], Jiao chenyang[1], SUN LIJUN[2], YU BIAO[2], Yan Shen[1], Wenjie Guo[1], and Qiang Xu[1]

[1]Nanjing University
[2]Shanghai Institute of Organic Chemistry

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Abstract

BACKGROUND AND PURPOSE Targeting AR-DBD is a potential strategy toward the treatment of CRPC, however, rational design of a small molecules targeting AR-DBD is still underdevelopment. EXPERIMENTAL APPROACH MST, ITC and other different assays has been used to confirm the binding of SBF-1 to AR, also CHIP has been used to confirm the blockade of AR binding to its target genes. The associated signaling pathway affected by SBF-1 has been identified by western blotting. Also, mutant AR-LBD and the the AR lacking DBD has led to the identification of the SBF-1 binding location in the AR. KEY RESULTS SBF-1 induced apoptosis and cell cycle arrest in both LNCaP and PC3/AR+ cell lines, also, inhibited the activation of the AR/IGF-1 and IGF1/AKT/FOXO1/PNCA pathways, which evidenced by decreased expression of p-AR, IGF-1, p-AKT, PCNA and Bcl-2. By using multiple methods, we found that SBF-1 could directly bind to AR and block the transcription of its target genes. Moreover, the interaction between SBF-1 and AR-DBD was confirmed, which overcame the re-activation of AR signaling by mutations in the AR-LBD. In the xenograft models of both ARWT and ARmutant prostate cancer, SBF-1 displayed a strong efficacy at very low doses including the inhibition of tumor growth, prolongation of survival time by inhibiting AR signaling. CONCLUSION AND IMPLICATIONS Our study here found a novel identified inhibitor of AR, SBF-1, for the first time, which is different from the current antiandrogens and may serve as a leading compound for the treatment of prostate cancer.

Ahmed Elgehama [a], Binglin Wang [a], Chenyang Jiao [a], Lijun Sun [c, d], Biao Yu [c], Yan Shen [a], Wenjie Guo[a, *], Qiang Xu [a, b, *]

[a] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, China.

[b] Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, 209 Tongshan Road, Xuzhou 221004, Jiangsu, China.

[c] State Key Laboratory of Bio-organic and Natural Products Chemistry, Shanghai Institute of Organic Academy, Shanghai 200032, China

[d] Department of Chemistry, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, China

Running title: SBF-1 binds AR-DBD and serves for the treatment of prostate cancer

[*]**Correspondence to:** Qiang Xu, Ph.D. or Wenjie Guo, Ph.D.

State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, 210023, China. Tel/Fax: +86-25-89687620; E-mail: molpharm@163.com (Q. Xu) or guowj@nju.edu.cn (WJ. Guo).

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Bullet point summary

What is already known

1. CRPC arise from AR-LBD point mutations
2. AR-DBD is feasible target of CRPC

What this study adds

1. Solid evidence on a reliable small molecule targeting AR-DBD
2. A total blockade of AR binding to its target genes, allowing a significant inhibition of CRPC cells.

Clinical significance

Over-coming CRPC in highly metastatic PCa with a significant low administering dose.

Abstract BACKGROUND AND PURPOSE Targeting AR-DBD is a potential strategy toward the treatment of CRPC, however, rational design of a small molecules targeting AR-DBD is still under development.**EXPERIMENTAL APPROCAH**MST, ITC and other different assays has been used to confirm the binding of SBF-1 to AR, also CHIP has been used to confirm the blockade of AR binding to its target genes. The associated signaling pathway affected by SBF-1 has been identified by western blotting. Also, mutant AR-LBD and the AR lacking DBD has led to the identification of the SBF-1 binding location in the AR.**KEY RESULTS**SBF-1 induced apoptosis and cell cycle arrest in both LNCaP and PC3/AR+ cell lines, also, inhibited the activation of the AR/IGF-1 and IGF1/AKT/FOXO1/PNCA pathways, which evidenced by decreased expression of p-AR, IGF-1, p-AKT, PCNA and Bcl-2. By using multiple methods, we found that SBF-1 could directly bind to AR and block the transcription of its target genes. Moreover, the interaction between SBF-1 and AR-DBD was confirmed, which overcame the re-activation of AR signaling by mutations in the AR-LBD. In the xenograft models of both ARWT and ARmutant prostate cancer, SBF-1 displayed a strong efficacy at very low doses including the inhibition of tumor growth, prolongation of survival time by inhibiting AR signaling.**CONCLUSION AND IMPLICATIONS**Our study here found a novel identified inhibitor of AR, SBF-1, for the first time, which is different from the current antiandrogens and may serve as a leading compound for the treatment of prostate cancer.**KEY WORDS:** Prostate cancer; SBF-1; androgen receptor; androgen receptor mutation; DNA binding domain, and ligand binding domain.**Abbreviations:** AR, androgen receptor; LBD, ligand binding domain; DBD, DNA binding domain; CRPC, castration resistant prostate cancer; IGF-1, insulin like growth factor 1.

Introduction

Prostate cancer is considered as one of the most common cancers in men. There are 174,650 estimated new cases in 2019 while 31,62 estimated deaths in USA (Siegel, Miller & Jemal, 2019). Although the etiology of prostate cancer remains unclear, aging and a familial history are the two most significant risk factors that increase a man's chances of developing the disease. Prostate cancers are reliant on male sex hormones called androgens for growth and survival. Androgens exert their effects throughout the body by binding to the androgen receptor (AR), a ligand inducible transcription factor that is critical not only for the growth and maintenance of the normal prostate, but also the development and progression of prostate cancer. Thus, manipulations of androgens in the body through lowering its levels or prevent androgens from binding to AR is considered as reliable strategy in the treatment of prostate cancer, which could effectively reduce

symptoms and tumor burden in almost all the patients. It is now accepted that despite various hormonal manipulations, the AR continues signaling throughout prostate cancer progression and remains the key therapeutic target. Androgens are crucial for the growth, development and maintenance of the prostate. The main androgen circulating in the male bloodstream is testosterone, which is converted via an enzyme called 5-α reductase to androgen dihydrotestosterone (DHT) in the prostate. Although similar in structure, DHT has a higher dissociation constant with AR (Deslypere, Young, Wilson & McPhaul, 1992; Dupaul-Chicoine et al., 2010; Kaufman & Pinsky, 1983). Like other hormone receptors, the exons of the AR code for functionally distinct regions of the protein. Exon 1 codes for the amino terminal transactivation domain (NTD), which plays a key role in transactivation, dimerization, and recruitment of co-regulator(s) involved in transcriptional function. Following NTD is the DNA binding domain (DBD) and ligand binding domain (LBD) of AR. The DBD of AR contains PBox recognition helix and DBox site that control DNA specificity and dimerization (Shaffer, Jivan, Dollins, Claessens & Gewirth, 2004). AR inside the nucleus binds to specific recognition sequences, androgen response elements (AREs), in the promoters and enhancers of target genes (Claessens, Denayer, Van Tilborgh, Kerkhofs, Helsen & Haelens, 2008). One of its main transcription products is insulin-like growth factor 1 (IGF-1), which acts on its receptor IGF-1R and activates MAPK and PI3K signaling to mediate cell proliferation and growth (Guntur & Rosen, 2013). The LBD of AR takes part in the posttranslational modifications of AR. The strong ligand-independent activation function 1 (AF1) in N terminus of AR interacts with the LBD. In response to ligand binding i.e. DHT, LBD becomes phosphorylated and triggers translocation of AR into the nucleus (Denayer, Helsen, Thorrez, Haelens & Claessens, 2010; Kuiper & Brinkmann, 1995; Schaufele et al., 2005; Wong et al., 2004).

From above findings, the hormonal therapy is usually conducted against prostate cancer to block AR signaling, either reducing androgen production or antagonizing AR. Current clinically used antiandrogens such as flutamide, bicalutamide, and enzalutamide mainly target the hormone binding pocket (HBP) that is located in the LBD of AR. However, the drug resistance rises from the fact that tumors often develop various mechanisms to reactivate androgen receptor signaling. This frequently involves sensitizing the tumor to low level of androgens through overexpression of AR, which in roughly 30% of castrate-resistant prostate cancer (CRPC) occur through amplification of the receptor [Linja, Savinainen, Saramäki, Tammela, Vessella & Visakorpi, 2001; Visakorpi et al., 1995]. Antiandrogens can lose their antagonism for AR and behave as partial agonists in the setting of AR overexpression [Chen et al., 2004]. Although mutation of AR does not seem to be common in early stages of prostate cancer, mutations have been well documented upon relapse in the setting of antiandrogen therapy [Gottlieb, Beitel, Nadarajah, Paliouras & Trifiro, 2012]. There has been a wide degree of difference in reports of incidence of AR mutations, but recent next gene sequencing studies confirmed that they occur in 20% of patients with metastatic CRPC (Beltran et al., 2013). These mutations often increase ligand promiscuity, allowing other endogenous androgens (or hormones) to activate AR signaling, and some of them can convert antiandrogens into agonists of the mutant receptor (Hara et al., 2003). The clinical observation of antiandrogen withdrawal syndrome, where prostate-specific antigen (PSA) levels decrease or tumors regress upon withdrawal from bicalutamide or flutamide treatment, correlates with the presence of AR mutations (Paul & Breul, 2000).

Recent work has also shown that expression of AR splice variants, that have distinct C-terminal extensions encoded by cryptic exons from the intron regions between canonical coding exons of AR, occurs in castration resistance (Hu et al., 2009). These variants often happen in the LBD and may exhibit constitutive AR activation. Although reports shown that these variants still require full-length AR to function, recent studies have suggested that expression of these variants is sufficient to elicit transcription of AR target genes in the absence of androgen and confers resistance to the novel antiandrogen enzalutamide (Li et al., 2013; Watson et al., 2010). As mentioned above, prostate cancer is continually undergoing AR mutations that switch the antiandrogen from antagonist to agonist and eventually relapses to lethal CRPC. To combat the mutation driven drug resistance, several rational antiandrogen design strategies have been developed, such as targeting HBP of AR (Tian, He & Zhou, 2015). However, it seems like ongoing running race and remains a big challenge.

In this study, we report a novel antiandrogen, a synthetic steroidal glycoside SBF-1, directly targeting AR-

DBD but not AR-LBD to exhibit a strong cytotoxicity towards two prostate cancer cell lines, LNCaP and PC3/AR$^+$ cells, with considerably low IC$_{50}$. This compound strongly attenuates IGF1/AKT/FOXO1/PCNA signaling, and eventually leads to apoptosis and cell cycle arrest. Interestingly, SBF-1 shows a significant binding to AR-DBD of whether the wild type AR or different mutant AR isoforms and blocks the interaction between the AR (AR mutants) with their target genes. SBF-1 is among such a handful developed antagonists against the binding of AR to the genes, in our knowledge, which is quite different from those blocking androgen-AR interaction, and can be considered as a potential compound for treating advanced prostate cancer cases with AR mutation.

Materials and methods

Reagents

SBF-1 is a steroidal glycoside, which is synthesized by the co-author Prof. Biao Yu, and its structure was shown in Fig. 1A. Primary antibodies (AR, rabbit monoclonal, Cat# 5153T, RRID:AB_10691711), (phosphorylated AKT$_{S473}$, rabbit polyclonal, Cat# 9271, RRID:AB_329825), (AKT1, rabbit monoclonal, Cat# 2938, RRID:AB_915788), (Phosphorylated FOXO1$_{S256}$, rabbit polyclonal, Cat# 9461, RRID:AB_329831), (FOXO1, mouse monoclonal, Cat# 97635, RRID:AB_2800285), (Bcl-2, mouse monoclonal, Cat# 15071, RRID:AB_2744528), (PCNA, rabbit monoclonal, Cat# 13110, RRID:AB_2636979) were purchased from Cell Signaling Technology (Beverly, MA). Primary antibody (Phosphorylated AR$_{S515}$,rabbit polyclonal, Cat# ab128250, RRID:AB_11141430) were purchased from Abcam (China). Primary antibody (IGF-1, rabbit polyclonal, Cat# PA1-86913, RRID:AB_2122127) were purchased from Thermo scientific. Primary antibodies (β-Actin, mouse monoclonal, Cat# sc-47778 HRP, RRID:AB_2714189), (GAPDH, mouse monoclonal, Cat# sc-32233, RRID:AB_627679) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were from Sunshine Biotechnology (Nanjing, China). 5α-Dihydrotestosterone (DHT) solution were purchased from Sigma-Aldrich (St. Louis, MO). All the plasmids: pcDNA3.1 [Flag-AR$_{WT}$, Flag-AR$_{[?]DBD}$, GFP- AR$_{WT}$, GFP- AR$_{L702H}$, GFP- AR$_{W742C}$, GFP- AR$_{F876L}$, and GFP- AR$_{T878A}$] were purchased from Gene-Script (Nanjing, China). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

LNCaP cells expressing AR with a novel mutation T878A in the AR ligand domain, PC3/AR$^+$ cells, a stable expressing AR$_{WT}$ cell line originally as a sub-line from the parental cells PC3, and human embryonic kidney cell line HEK293T were obtained from the Chinese Academy of Medical Sciences (Tianjin, China). All the cells were maintained in DMEM medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 U/ml penicillin, and 100 mg/ml streptomycin.

MTT assay

1×10^5 cells were seeded into 96-well plates, and incubated with various concentrations of SBF-1 for indicated time periods. The survival rate was detected as described previously (Li et al., 2012).

Apoptosis, cell cycle and cell adhesion assay

Cell apoptosis was determined by annexin V/PI staining. In brief, the cells were measured by flow cytometry after addition of FITC-conjugated annexin V and PI as previously described (Azeem et al., 2017). Annexin V$^+$ / PI$^-$ and annexin V$^+$ / PI$^+$ were considered as apoptotic cells in the early and late phase, respectively. Samples were analyzed by flow cytometry on a FACScan (Becton Dickinson).

For cell cycle analysis, cells were stained with propidium iodide (PI) as described in (Sun et al., 2010b), and the cell cycle distribution was analyzed by flow cytometry on a FACScan (Becton Dickinson). The percentages of cells in G0/G1, S, and G2 phases were counted and compared.

Cell adhesion assay was performed as reported (Li et al., 2012).

Western blot

Cells were lysed in radio immunoprecipitation assay (RIPA) lysis buffer as described previously (Li et al., 2014). The whole cell lysates were collected and separated by 10% SDS-PAGE and subsequently electro-transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The blocked membrane was incubated with the indicated antibodies. Final detection was performed using a chemiluminescent substrate system (Cell signaling, CA).

RNA extraction, reverse transcription PCR (RT-PCR) and ChIP-PCR analysis

Cells were collected and lysed in Trizol (Takara, Tokyo, Japan). RNA samples were used for reverse transcription with Oligo (dT) primers (Takara, Tokyo, Japan). The cDNA products were subjected to RT-PCR. cDNA amplification was performed for 35 cycles (94 degC for 30 s, 58 degC for 30 s and 72 degC for 30 s) using Tag DNA polymerase (Promega, Shanghai, China). The RT-PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The Gel Imaging and Documentation DigiDoc-It System (version 1.1.23; UVP, Inc., Upland, CA) was used to scan the gels. Gapdh was performed as a loading control. The primer sequences used in this study were as follows:

IGF-1: forward, 5'-GCTCTTCAGTTCGTGTGTGGA-3';

reverse, 5'-GCCTCCTTAGATCACAGCTCC-3';

PCNA: forward, 5'-CCTGCTGGGATATTAGCTCCA-3';

reverse, 5'-CAGCGGTAGGTGTCGAAGC-3';

PCNA: forward, 5'-AGGCACTCAAGGACCTCATCA-3';

reverse, 5'-GAGTCCATGCTCTGCAGGTTT-3';

β-actin: forward, 5'-CATGTACGTTGCTATCCAGGC-3';

reverse, 5'-CTCCTTAATGTCACGCACGAT-3'.

For ChiP primers, they were as follows:

IGF-1:forward, 5'-CAGGTCTGGCTCATTTCCATC-3'; reverse, 5'-GCGCTTTCCATGGCTGTC-3'; probe, 6FAM-CCCCTGGGAAAGCACACCTGGA; PCNA: forward, 5'-CCACCATAAAGCTGGGGCTT-3'; reverse, 5'-TCTCCCCGCCTCTTTGACTC-3'.

probe, 6FAM-CCCCTGGGAAAGCACACCTGGA

Gene silencing

PC3 or HEK293T cells were seeded into 6 wells plate at a confluence of 1×10^6 and then transfected with the siRNA using Lipofectamin RNAi MAX (Thermo Scientific) according to the manufacturer protocol, the following sequences used for siRNA as follows:

FOXO1: FOXO1 (sense: 5'-GGAGGUAUGAGUCAGUAUAUU-3'),

AKT1: AKT1 (sense: 5'-UGCUGUUGACAGUGAGCG-3'),

IGF-1: IGF-1 (sense: 5'-CGCAGGUAACGAUGGGAAAUU-3'),

Control sequence (sense: 5'-UGCCGUUCUUCAACGAGGA-3').

The siRNA oligonucleotides, together with the corresponding antisense oligonucleotides, were synthesized by Gene Script (Nanjing, China).

Electrophoretic mobility shift assay (EMSA) and determination of Kd values

PC3 cells were transfected with AR_{WT} or $AR_{[?]DBD}$ expression plasmid. Cell extract (5 μg) collected 36 h after transfection was incubated with γ32P ATP-labeled oligonucleotide probes with either AR_{WT} or $AR_{[?]DBD}$ consensus binding sites (Santa Cruz Biotechnology) in a buffer containing 20 mM HEPES, pH 7.9,

50 mM KCl, 0.1 Mm EDTA, 2 mM MgCl$_2$, 2 mM spermidine, 0.5 mM dithiothreitol, 1 µg dI-dC and 10 % glycerol for 20 minutes at room temperature, followed by resolution of complexes on 7% native PAGE. For super-shift analysis, antibody against AR from Cell Signaling Technologies was used (Hellman & Fried, 2007).

The dissociation constants of the protein-DNA complexes were measured under equilibrium binding conditions. The volumes of the bands corresponding to free and bound DNA were quantified using ImageQuant software (version 5.2). The bound-DNA fraction (ϑapp) was calculated as the volume of the band corresponding to the bound DNA, divided by the sum of the volumes of the bands corresponding to free and bound DNA. Data were fit to a modified two-state binding equation to determine apparent dissociation constants for each protein-DNA complex as previously reported (Bird, Lajmi & Shin, 2002).

SBF-1/DHT competitive assay

Flag-AR$_{WT}$ construct was transfected into PC3 cells and the total protein extract was obtained and purified along with Flag tag removal using enterokinase enzyme as reported (Skala, Goettig & Brandstetter, 2013). SBF-1 was checked whether being binding to the ligand binding domain of AR (AR-LBD) or not by a competitive assay using substrate Fluormone AL Green (Thermo Fisher) according to manufacture protocol.

Micro scale thermophoresis (MST)

GFP-AR constructs were purchased from Gene-Script (China). Each construct was transfected into PC3 cells and the total protein was obtained in MST buffer followed by addition of SBF-1 in serial dilution of 16 folds. The assay was performed as reported (Wienken, Baaske, Rothbauer, Braun & Duhr, 2010) and the binding affinity was checked using Monolith NT.115.

Isothermal titration calorimetry (ITC)

Flag-AR$_{WT}$ and Flag-AR$_{[?]DBD}$ was expressed and purified (Zhou, Suino-Powell, Ludidi, McDonnell & Xu, 2010), along with the removal of the Flag tag (Skala, Goettig & Brandstetter, 2013). Then the binding affinity between SBF-1 and both isoforms was checked using MicroCal ITC 200 (Duff, Grubbs & Howell, 2011).

Luciferase activity and DNA pull-down assay

IGF-1 and PCNA constructs consisting of 1-3000 bp of each gene were purchased from Gene-Script (China). The assay was performed as described in Fig. S2A.

Animal models and drug administration

Male BALB/c nude mice (6-8 week-old) were provided by Jiangsu Gempharmatech co. Ltd. (Nanjing, China). The mice were kept under pathogen-free conditions in type IV Makrolon cages (6 mice per cage) in an air flow cabinet at 23°C, 12 h/12 h day/night cycle. Sterilized food and sterilized acidified water have been provided to the animals regularly. 1×10^6 LNCaP and PC3/AR$^+$cells were injected subcutaneously into the right flank. After 6 days of injection, all mice formed tumors and the day was marked as day 0. Vehicle (0.05% DMSO in PBS), 10 and 30 µg/kg SBF-1 were intraperitoneally injected to every group respectively every day since day 0 for 45 days. At the same time, body weights and tumor volumes were measured. For tumor growth assay, mice were euthanized 14 days after drug treatment, and the weight of tumor was measured. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of Nanjing University. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

In Silico analysis

Autodock vina 4.2 was used in the analysis of SBF-1 docking to the AR-DBD (PDB ID: 1R4I). Out of the many docking poses, only those possessing the highest docking score were chosen which gave a binding affinity at [-11.3 kcal/mol]. When we analyzed the predicted binding affinity, the large absolute value of the

energy means the great affinity of corresponding ligand-receptor reaction as this datum represents the free energy of binding in AutoDock Vina v4.2 docking software.

Statistical analysis

Data were expressed as means $\pm$ SEM. The Student's t test was used to evaluate the difference between groups. $P < 0.05$ was considered significant. All in vitro data were generated from at least three independent experiments.

Results

SBF-1 showed a strong cytotoxicity against both AR_{WT} and AR_{mutant} prostate cancer cells

LNCaP cells (expressing AR with a novel mutation T878A in the AR ligand domain) and $PC3/AR^+$ (PC3 cells with stable AR_{WT} expression) were incubated with different concentrations of SBF-1 for 3 h. As the result, SBF-1 showed very strong cytotoxicity towards LNCaP and $PC3/AR^+$ cells (Fig. 1B with IC_{50} values).

After incubated with different doses of SBF1, cells were collected and subjected to analysis of adhesive ability to fibronectin and laminin. SBF-1 showed a significant inhibition on adhesive ability of LNCaP and $PC3/AR^+$ cells to fibronectin and laminin in a concentration-dependent manner (Fig. 1C).

Next, the apoptosis assay and cycle arrest analysis were shown in Fig. 1D-G. As compared with the control, the percentage of apoptotic cells was significantly increased, and the cell cycle arrest was found in G1 and G2/M phase in SBF-1-treated LNCaP and $PC3/AR^+$cells

SBF-1 downregulated the AR/IGF1 and its subsequent IGF-1/AKT/ FOXO1/PCNA signaling via directly binding to AR

Considering the important role of AR signaling in the growth of prostate cancer cells, we next examined the effect of SBF-1 on the signal of AR/IGF-1 and its subsequent IGF-1/AKT/FOXO1/PCNA. As shown in Fig. 2A, the expressions of IGF-1, PCNA, Bcl-2, pAR_{S515}, $pAkt_{S473}$ and $pFOXO1_{S256}$ were greatly decreased in both LNCaP and $PC3/AR^+$ cells by SBF-1 without affecting the total AR, AKT1, and FOXO1 expressions. This result suggests that SBF-1 downregulated AR/IGF-1 and IGF-1/AKT/FOXO1/PCNA pathways. It should be noted that the activation but not the expression of key enzymes or transcription factors was inhibited in the effect of SBF-1, which hints that the target of SBF-1 may be in the up-stream of AR signaling. To further confirm the effect of SBF-1 on the signaling, we used DHT to stimulate AR. As the result, DHT greatly increased the AR expression as well as the AR phosphorylation and the expressions of products IGF-1 and PCNA in both pathways. Against this, SBF-1 greatly blocked DHT-increased expressions of AR, pAR_{S515}, IGF-1, and PCNA in both AR_{WT} and AR_{mutant} cells (Fig. 2B). This result indicates dual effects of SBF-1 on AR/IGF1 axis and their down-stream signaling. Also, as seen in Fig. 2C, SBF-1 strongly suppressed the mRNA expression of IGF-1 and PCNA in either DHT presence or not. Especially in the case that DHT doubled the IGF-1 mRNA expression, SBF-1 still showed an almost complete inhibition. These findings suggest a possibility of SBF-1 to directly block the gene transcription mediated by AR.

To examine how SBF-1 affects AR and its subsequent signaling, we hypothesized that SBF-1 might directly bind to AR since it is a steroidal glycoside. We first conducted MST technique to examine whether there is any binding between SBF-1 and AR. The purified AR_{WT} was tagged with GFP tag and transfected into HEK293T cells and the total cell lysis was extracted and incubated with different doses of SBF-1 to determine the binding affinity between SBF-1 and AR. As shown in Fig. 2D, the binding affinity between SBF-1 and AR is strong enough to conclude the binding, and the measured affinity was about 321 nM. Furthermore, we used ITC technique to confirm the binding between SBF-1 and AR. Namely, the purified AR_{WT} was used along with SBF-1, and there is actually a strong binding with 2.95×10^{-5} M of the detected [?]K for the reaction between SBF-1 and AR_{WT} protein (Fig. 2E).

Then, the polarity shift assay was performed to check whether the ligand-binding domain (AR-LBD) is the binding site of SBF-1. Flag-AR_{WT} was transfected into HEK293T and then purified with the cleavage of

Flag tag and incubated with fluorescent substrate along with the addition of SBF1 or DHT. When monitored as a fluorescent reading output, a significant shift in the polarization was detected to reveal a competitive replacement of substrate binding to AR-LBD by DHT, which confirmed the direct binding of DHT to the AR-LBD. However, there was no shifting signal in the case of SBF-1, which reveals that SBF-1 dose not bind to the AR-LBD at all (Fig. 2F). Above findings suggest that SBF-1 binds to the AR protein but AR-LBD is not the binding site.

SBF-1 bound to AR mutants and inhibited their activation

AR signaling is known to be caused in castrate-resistant prostate cancer (CRPC) by activating AR point mutations. The most frequently identified AR point mutations include T878A (Fenton et al., 1997; Taplin et al., 1999; Taplin et al., 1995; Veldscholte et al., 1990), W742C(Lallous et al., 2016; Steketee, Timmerman, Ziel-van der Made, Doesburg, Brinkmann & Trapman, 2002; Tan et al., 1997; Taplin et al., 2003; Watson, Arora & Sawyers, 2015; Yoshida et al., 2005), L702H(Lallous et al., 2016; Steketee, Timmerman, Ziel-van der Made, Doesburg, Brinkmann & Trapman, 2002; Tan et al., 1997; Taplin et al., 2003) and F876L mutations (Korpal et al., 2013). According to these findings, we covered these frequently occurring mutations in CRPC through the construction of AR mutants to examine the effect of SBF-1 on those mutants (Fig. 3). The mutants tagged with GFP tag were transfected into PC3 cells. After 36 h of transfection, the cell lysate from transfected PC3 cells were collected to examine the binding with SBF-1. MST was used to detect the binding affinity between SBF-1 and the AR mutants. As the result, SBF-1 showed a strong binding to all of the mutants with the affinity 10 μM to L702H, 610 nM to W742C, 1.9 μM to F876L, and 385 nM to T878A, respectively (Fig. 3A). Also, we examined the activity of those mutants and their wild type in the presence of DHT stimulation. We used SBF-1 and DHT alone or both to treat the cells. As the result, the stimulation of AR wild type and mutants with DHT (0.1 and 10 nM) caused an increase in the luciferase activity of AR wild type and its mutants, while in case of SBF-1, there was no change in the activity of neither the wild type nor the mutants. Surprisingly, the combined treatment of DHT and SBF-1 did not cause any change in the luciferase activity, suggesting that SBF-1 could block AR activity increased by DHT due to its binding to it (Fig. 3B). We examined the effect of SBF-1 on the phosphorylation state of the constructed AR_{WT} and the mutants. After 36 h of transfection of GFP tagged constructs, the PC3 cells were treated with or without DHT or a combination of SBF-1 and DHT for 6 h. As the result, SBF-1 markedly reduced the phosphorylation of AR and its mutants even in the presence of DHT (Fig. 3C).

SBF-1 may occupy a novel binding site AR-DBD for blocking the interaction between the transcription factor and its target gene

Above results suggest that SBF-1 may have a novel binding site in AR. To find the site, we constructed an ARE-1 sequence, which known to be a consensus recognition site for the AR (Denayer, Helsen, Thorrez, Haelens & Claessens, 2010). This constructed sequence was incubated with each purified AR_{WT} or $AR_{[?]DBD}$ (Androgen receptor lacking DBD). As the result, there is a shift band in case of AR_{WT} but not in $AR_{[?]DBD}$ with the Kd values of 370 nM in AR_{WT} and N/A in $AR_{[?]DBD}$, respectively (Fig.4A), suggesting that the AR-DBD is a potential target site of SBF-1. We further analyzed the binding affinity between SBF-1 and $AR_{[?]DBD}$. By adding 16 different doses of SBF-1 to PC3 cells transfected with the previously constructed and GFP-tagged $AR_{[?]DBD}$, a very low binding affinity of SBF-1 with $AR_{[?]DBD}$ was calculated as 698 μM (Fig. 4B). Furthermore, we expressed and purified $AR_{[?]DBD}$ and used ITC technique to determine the thermodynamic parameters of the interaction between SBF-1 and $AR_{[?]DBD}$, the result shows that SBF-1 totally failed to show any binding signal with the purified $AR_{[?]DBD}$ (Fig. 4C). Structural analysis revealed the existence of SBF-1 and AR-DBD binding (Fig. S1A). We further analyzed the gene expression enrichment related to the AR through ChIP-qPCR assay, and used AR ChiP grade antibody to determine the IGF-1 enrichment status as shown previously in Fig. 2A and B. We confirmed that IGF-1 was reduced due to the treatment of SBF-1. In Fig. 4E, we found that DHT induced a significant increase in the AR target gene IGF-1 enrichment while SBF-1 completely inhibited this enrichment. SBF-1 also inhibited the PCNA gene expression. Computer docking and DNA pull down assays proved this result (Fig. S1 and S2). These results suggest that SBF-1 may directly block AR from binding to target genes by occupying its DBD.

SBF-1 almost completely blocked the activation of AR and its subsequent signaling by DHT

It is known that activation of IGF-1 through glucose uptake will cause the activation of AKT-FOXO1 axis (Iwamura, Sluss, Casamento & Cockett, 1993; McKeehan, Adams & Rosser, 1984). When the prostate cancer cells were treated with 20 mM Glucose, 200 nM SBF-1 and 5 nM DHT or both SBF-1 and DHT for 3 h, SBF-1 decreased the protein levels of IGF-1, PCNA, $pAKT_{S473}$ and pFOXO1 in the presence of Glucose or DHT (Fig. 5A and B), as well as the mRNA level of IGF-1 increased by DHT and glucose (Fig. 5C). In Fig. 5D and E, we silenced the IGF-1 and FOXO1, respectively in both LNCaP and PC3/AR^+ cells. SBF-1 and DHT showed an opposite effect on the expressions of the downstream signal proteins PCNA and Bcl-2. When they were used together, SBF-1 almost completely blocked the DTH-increased expressions of PCNA and Bcl-2. These results suggest that SBF-1 may display its anti-PCa activity despite the endogenous levels of androgens and AR mutation.

SBF-1 strongly inhibited the growth of both AR_{WT} and AR_{mutant} prostate cancer in nude mice

Finally, the effect of SBF-1 on prostate cancer growth *in vivo* was tested. Nude mice were injected s.c. with LNCaP or PC3/AR^+ cells. After the tumor reached 50 mm^3, SBF-1 was administered i.p. at 10 and 30 μg/kg. SBF-1 significantly reduced the tumor size in mice, in a dose-dependent manner (Fig. 6A and B). At the end of treatment, animals were injected with ICG dye-conjugated IGF-1 antibody and after 3 hours from injection, the tumor size and the fluorescent intensity were measured. SBF-1 dramatically reduced both tumor size and the fluorescent intensity of IGF-1 in mice at such very low doses in a dose-dependent manner, suggesting a relation of the strong inhibition of tumor growth to the reduced IGF-1 expression (Fig. 6C-F). In this case, SBF-1 significantly avoided the decrease in body weights of mice bearing tumor (Fig. 6G and H) and prolonged the survival rate (Fig. 6I and J). In addition, SBF-1 inhibited the expressions of pAR_{S515}, $pAKT_{S473}$, $pFOXO1_{S256}$, IGF-1, PCNA, and Bcl-2 proteins extracted from the tumor tissues in both models (Fig. 6K and L).

Discussion

The present study realized a specific blockade of the AR and its target gene IGF-1, which induces both apoptosis and cell cycle arrest by a novel antiandrogen SBF-1. This unique compound was used for the treatment of prostate cancer in a xenograft model. As we know, AR is a hormone inducible transcription factor, which drives expression of tumor promoting genes and represents an important therapeutic target in prostate cancer (Dalal et al., 2018). Higher expression of AR in prostate cancer with shorter overall survival was found in patients (Fig. S3). Currently, small molecule drugs used in the treatment of prostate cancer mainly interfere with steroid recruitment to prevent AR-driven tumor growth (Caboni & Lloyd, 2013; Lallous, Dalal, Cherkasov & Rennie, 2013). However, those kinds of small molecules are rendered ineffective in the advanced prostate cancer by emergence of LBD mutations or expression of constitutively active variants such as AR-V7 that lack the LBD. As the cell lines of prostate cancer, LNCaP cells express AR with a novel mutation T878A in the AR-LBD, which is similar to human prostatic adenocarcinoma, and their growth can be inhibited by androgen withdrawal. Another cell line PC3/AR^+ cells have a stable expression of AR_{WT}, which is originally a sub-line from the parental cells AR negative PC3 cells. By using these two cell lines, we aimed at finding a novel inhibitor against prostate cancer in both AR_{WT} and AR_{mutant} levels through targeting AR.

As the result, SBF-1 strongly inhibited proliferation of both LNCaP and PC3/AR^+ cells, suggesting a possibility that this compound can be used for the treatment of prostate cancer with both AR_{WT} and AR_{mutant}. For investigating the mechanisms underlying its effect, we next found that SBF-1 showed a significant inhibition on the adhesive ability of both LNCaP and PC3/AR^+ cells to fibronectin and laminin, increased the percentage of apoptotic cells, and caused the cell cycle arrest in G1 and G2/M phase.

AR can be activated by the binding of endogenous androgens, including testosterone and DHT (Gao, Bohl & Dalton, 2005). The AR mediates the growth of benign and malignant prostate in response to DHT. In patients undergoing androgen deprivation therapy for prostate cancer, AR drives prostate cancer growth despite low circulating levels of testicular androgen and normal levels of adrenal androgen (Mohler et al.,

2011). Usually, prostate cancer cells gradually lose their dependence on androgen signaling through the AR and become resistant to hormonal therapy. It is now established that throughout various hormonal manipulations, castrate-resistant prostate cancers continue to express the AR (Hobisch, Culig, Radmayr, Bartsch, Klocker & Hittmair, 1995; Hobisch, Culig, Radmayr, Bartsch, Klocker & Hittmair, 1996; Sadi & Barrack, 1991; Tilley, Lim-Tio, Horsfall, Aspinall, Marshall & Skinner, 1994; van der Kwast et al., 1991) and IGF-1 as the transcription product of AR has been shown to independently activate the AR in the absence of DHT, with a mechanism that involves downstream phosphorylation of either the AR or its associated proteins (Culig et al., 1994; Gregory et al., 2004). It has also been reported that inhibition of IGF-1 could suppress prostate cancer cell growth (Zheng et al., 2012). On the other hand, IGF-1 will also activate its downstream proteins such as AKT kinase, which regulates the cell proliferation and survival. AKT is usually activated by hormones, growth factors, and chemical drugs (Zheng et al., 2015; Zheng et al., 2012; Zhu et al., 2015). The downstream forkhead transcription factor family FOXO plays a vital function in cell apoptosis and survival in variety of cell types, which could be phosphorylated by AKT kinase (Tzivion, Dobson & Ramakrishnan, 2011). Those findings inspired us to test the effect of SBF-1 on the AR signaling while stimulated with DHT. As shown in Fig. 2A, the expressions of IGF-1, PCNA, Bcl-2, pAR_{S515}, $pAKT_{S473}$ and $pFOXO1_{S256}$ were greatly decreased in both LNCaP and PC3/AR$^+$ cells by SBF-1 without effects on the AR, AKT1, and FOXO1 expressions. This result suggests that SBF-1 may downregulate both AR/IGF-1 and IGF1/AKT/FOXO1/PCNA pathways. When we used DHT to stimulate AR, DHT greatly increased the AR expression as well as the AR phosphorylation and the expressions of downstream proteins IGF-1 and PCNA. Against this, SBF-1 down-regulated DHT-increased expressions of AR, pAR_{S515}, IGF-1, and PCNA in both cell lines (Fig. 2B). This result indicates a dual effect of SBF-1 on AR/IGF1 axis and their down-stream signaling. SBF-1 also strongly suppressed the mRNA expression of IGF-1 and PCNA even in the case of DHT-doubled expression (Fig. 2C). These findings suggest a possibility of SBF-1 to directly block the gene transcription mediated by AR that is known to bind the promoter of its target genes.

To examine how SBF-1 affects AR and its subsequent signaling pathways, we hypothesized that SBF-1 might directly bind to AR since it is a steroidal glycoside. Both MST and ITC assays were used to examine the binding affinity between SBF-1 and purified AR$_{WT}$. After a strong binding was concluded (Fig. 2D, and E), we performed the polarity shift assay to check whether the AR-LBD is the binding site of SBF-1 because AR-LBD is recognized as the vital domain to be targeted in prostate cancer therapy (Wang et al., 2006). However, despite the significant binding to AR-LBD by DHT as a positive control, what we hypothesized for the binding of SBF-1 to AR-LBD is absolutely negative (Fig. 2F). Above findings suggest that SBF-1 does bind to the AR protein but AR-LBD is not the binding site.

AR signaling in CRPC tumor epithelial cells could be caused by activating AR point mutations. Such mutations are rare in untreated PC, but detected in 15–20% of CRPC patients (Grasso et al., 2012; Robinson et al., 2015; Taylor et al., 2010), and in up to 40% of CRPC patients treated with AR antagonists (Balk, 2002). Activating AR point mutations generally affects the c-terminal LBD, while about one-third occur in the transactivating NTD (Gottlieb, Beitel, Nadarajah, Paliouras & Trifiro, 2012; Steinkamp et al., 2009), resulting in broaden ligand specificity. The first and most frequently identified AR point mutation is the flutamide-driven T878A mutation (Fenton et al., 1997; Taplin et al., 1999; Taplin et al., 1995; Veldscholte et al., 1990), while W742C also has been reported after treatment with first-generation AR antagonists (Lallous et al., 2016; Steketee, Timmerman, Ziel-van der Made, Doesburg, Brinkmann & Trapman, 2002; Tan et al., 1997; Taplin et al., 2003; Watson, Arora & Sawyers, 2015; Yoshida et al., 2005). The T878A and L702H mutations have been observed in CRPC patients during abiraterone treatment (Lallous et al., 2016; Steketee, Timmerman, Ziel-van der Made, Doesburg, Brinkmann & Trapman, 2002; Tan et al., 1997; Taplin et al., 2003). Also, F876L mutation confers an antagonist-to-agonist switch that drives phenotypic resistance (Korpal et al., 2013). Taken together, we decided to cover these frequently occurring mutations in CRPC through the construction of AR mutants as shown in (Fig. 3) and determined the effect of SBF-1 on those mutants. As the result, SBF-1 showed a strong binding to all the mutant constructs, L702H, W742C, F876L and T878A (Fig. 3A). It should be emphasized that SBF-1 showed an almost complete inhibition against the DHT-increased risen activity of AR$_{WT}$ and the mutants (Fig. 3B). Above results suggest that

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SBF-1 may have a novel binding site of SBF-1 on AR.

Growing evidence suggests that castration resistance of prostate cancer may be partially mediated by AR splice variants lacking the LBD coding sequence, which leaves only the NTD and DBD as viable domains that are targetable by small molecules (Dehm, Schmidt, Heemers, Vessella & Tindall, 2008; Guo et al., 2009; Hu et al., 2009; Sun et al., 2010a; Watson et al., 2010). Inhibition of splice variant transcriptional activity would be a significant breakthrough in the development of a new class of anti-AR drugs (Dalal et al., 2014). To find the binding site of SBF-1 in AR, we constructed an ARE-1 sequence, which known to be consensus recognition site for the AR (Denayer, Helsen, Thorrez, Haelens & Claessens, 2010). This constructed sequence was incubated with each purified AR_{WT} or $AR_{[?]DBD}$ (Androgen receptor lacking DBD). As the result, SBF-1 did show a binding to AR_{WT} but not $AR_{[?]DBD}$ (Fig. 4A, and B), suggesting that AR-DBD might be the target domain for SBF-1. The ITC technique confirmed that SBF-1 totally failed to show any binding signal with the purified $AR_{[?]DBD}$ (Fig. 4C). To understand the result of SBF-1 binding to AR-DBD, furthermore, the AR-induced gene expression enrichment assay demonstrated that DHT induced a significant increase in the AR target gene IGF-1 enrichment while SBF-1 diminished this enrichment (Fig. 4E). These results suggest that SBF-1 might block AR from binding to its target genes by binding to its DBD.

The binding between SBF-1 and AR or its mutants leads to further insight into where could SBF-1 binds to AR, and especially all the found mutants are located in AR-LBD. This hints us that the current efforts for the treatment of prostate cancer to target AR may become resistant after the mutation accompanying with the malignant progression, and a novel inhibitor targeting the new domain of AR is needed. To our knowledge, a small molecule EPI-001 was reported to block transactivation of the NTD and was specific for inhibition of AR without attenuating transcriptional activities of related steroid receptors (Andersen et al., 2010). On the other hand, targeting AR-DBD may be a new strategy for CRPC treatment (Dalal et al., 2014). However, there is still little development of inhibitors that specifically target the NTD or DBD of the AR (Caboni & Lloyd, 2013; Lallous, Dalal, Cherkasov & Rennie, 2013). Here we propose a solid proof of strong anti-tumor small molecule SBF-1 that inhibit the growth of advanced prostate cancer through the binding to AR-DBD. That is why SBF-1 can bind both AR and its multiple mutants for the inhibition of both AR_{WT} and AR_{mutant} cells. In fact, IGF-1/AKT/FOXO1 axis has been known to be of importance in prostate cancer castration resistance. Stimulation of AR through androgens, i.e. DHT has shown to activate the antiapoptotic IGF-1/AKT/FOXO1/PCNA pathway in LNCaP and PC3/AR$^+$ cells (Zhao, Tindall & Huang, 2014). The activation of IGF-1/AKT/FOXO1/PCNA signaling is critical for mediating cell survival and involved in the castration resistance through the modulation of AR expression and its down-stream signaling. In this study, we used LNCaP cells that express AR, including the common AR mutation, ETV1 overexpression, and PTEN deletion, which serves as a good model to examine late-stage prostate cancer with metastatic potential, and also, PC3/AR$^+$ cells that express AR but WT and has normal PTEN expression (Kim, Park & Dong, 2006). These two cell lines will give a broad spectrum of how mutant prostate cancer cases could be affected by the treatment of SBF-1.

Currently, targeting AR-DBD has become increasingly in-focus as the AR-DBD exists in all forms of AR and its mutants, also helps in directly treating castration resistance in prostate cancer (Dalal et al., 2018). Blocking AR from regulating its target gene IGF-1 could help overcome castration resistance, and stimulation of IGF-1 using glucose will lead to the over-expression of IGF-1, which could explain how SBF-1 works. SBF-1 almost completely overcame the activated IGF-1 signaling through glucose intake or DHT stimulation (Fig. 5). This characteristic suggests that SBF-1 may have different mode of actions from the current agents against prostate cancer despite the endogenous levels of androgens and AR mutation, which is quite beneficial to various situations of PCa patients. Finally, such unique mechanism of SBF-1 was tested on prostate cancer growth in nude mice, where SBF-1 significantly reduced the tumor size in mice bearing either AR_{WT} or AR_{mutant} cells, and prolonged the survival rate at very low doses in a dose-dependent manner, with a strong decrease in the IGF-1 protein and its downstream signaling but without loss of body weights of tumor-bearing mice (Fig. 6). These findings provided great advantages for the targeting of AR-DBD against AR wild type and mutant prostate cancer by SBF-1.

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In summary, we present a novel antiandrogen targeting AR-DBD, for the first time, which attenuates AR in a wide spectrum of different variants for the treatment of prostate cancer especially the castration resistant prostate cancer (summarized in Fig. 7). Our findings suggest a better strategy in dealing with the development of advanced prostate cancer by targeting AR-DBD rather than the conventional methods.

Author contributions

Ahmed elgehama, designed and performed the experiments, along with data analysis. Binglin Wang, and Chenyang Jiao helped in the organization of the manuscript. Lijun Sun, and Biao Yu synthesized SBF-1. Yan Shen, supported the research. Wenjie Guo, and Qiang Xu corresponding authours.

Conflict of interest statement

The authors have declared no conflict of interest.

Declaration of transparency and scientific rigor Our Declaration addresses that this work followed a strict principle for transparent reporting and scientific precision of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Animal Experimentation, and as recommended by our university and lab.

Appendices: Supplementary figures 1-3.

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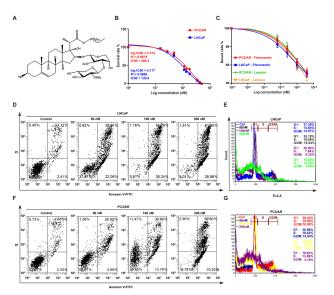
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Fig. 1. SBF-1 inhibited proliferation and adhesion, and induced apoptosis and cell cycle arrest in LNCaP and PC3/AR⁺ PCa cells. (A) Chemical structure of SBF-1. 1×10^5 LNCaP or PC3/AR⁺ cells were seeded into 96-well microplates, and incubated with various concentrations of SBF-1 for 24 h. Cell viability was determined by MTT assay (B). Cell adhesive ability was tested toward fibronectin and laminin (C). The percentages of apoptotic cells were determined by Annexin V/PI staining (D, F). The cell cycle was determined by PI staining (E, G). Values in B and C were shown as the mean ± SEM. Data in D-G were representative of three independent experiments.

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