Investigational New Drugs (2021) 39:442–457
https://doi.org/10.1007/s10637-020-01050-w

PRECLINICAL STUDIES

Selective targeting of the androgen receptor-DNA binding domain by the novel antiandrogen SBF-1 and inhibition of the growth of prostate cancer cells

Ahmed Elgehama[1] · Lijun Sun[2,3] · Biao Yu[2] · Wenjie Guo[1] · Qiang Xu[1,4]

Received: 18 September 2020 / Accepted: 10 December 2020 / Published online: 7 January 2021

Summary

Prostate cancers are reliant on androgens for growth and survival. Clinicians and researchers are looking for potent treatments for the resistant forms of prostate cancer; however, a handful of small molecules used in the treatment of castration-resistant prostate cancer have not shown potent effects owing to the mutations in the AR (Androgen Receptor). We used SBF-1, a well-characterized antitumor agent with potent cytotoxic effects against different kinds of cancers and investigated its effect on human prostate cancer. SBF-1 substantially inhibited the proliferation, induced apoptosis, and caused cell cycle arrest in LNCaP and PC3/AR$^+$ prostate cancer cell lines. SBF-1 inhibited the activation of the IGF-1-PNCA pathway, as demonstrated by decreased expression of IGF-1 (insulin-like growth factor 1), proliferating cell nuclear antigen (PCNA), and its downstream Bcl-2 protein. Using microscale thermophoresis (MST) and isothermal titration calorimetry (ITC) assays, we observed a direct binding of SBF-1 to the AR. SBF-1 binds to the AR-DBD (DNA-binding domain) and blocks the transcription of its target gene. SBF-1 demonstrated a potent antitumor effect in vivo; it inhibited AR signaling and suppressed tumor growth in animals. Our study suggests that SBF-1 is an inhibitor of the AR and might be used in the treatment of prostate cancer.

Keywords Prostate cancer · SBF-1 · Androgen receptor · Androgen receptor mutation · DNA binding domain

Introduction

Prostate cancer is one of the most commonly observed cancers among men. Approximately 174,650 new cases were observed in 2019 with 31,62 estimated deaths in the USA [1]. Prostate cancers are reliant on male sex hormones (androgens) for growth and survival. Similar to other hormone receptors, the androgen receptor (AR) exons code for functionally distinct regions within the AR protein, the amino-terminal transactivation domain (NTD), the DNA binding domain (DBD), the hinge region, and the ligand-binding domain (LBD). AR-DBD contains a PBox recognition helix and DBox sites that control DNA specificity and dimerization [2]. The AR binds to specific recognition sequences, androgen response elements (AREs) in the promoters and enhancers of its target genes inside the nucleus [3]. Insulin-like growth factor 1 (IGF-1) is a primary transcription product of AR that binds to its receptor IGF-1R and activates MAPK and PI3K signaling to mediate cell proliferation and growth [4]. The AR-LBD participates in the posttranslational modifications of the AR. In response to binding of ligands such as DHT, the LBD is phosphorylated and triggers the translocation of AR into the nucleus [5–8].

The current antiandrogens with clinical applications such as flutamide, bicalutamide, and enzalutamide mainly target the hormone-binding pocket (HBP) of the AR-LBD. However, since tumors often develop various mechanisms to reactivate androgen receptor signaling, such as via mutations, drug resistance is observed.

✉ Qiang Xu
molpharma@163.com

[1] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 200032, China

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

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

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

Drug resistance frequently involves sensitizing the tumor to a low level of androgens via AR overexpression; approximately 30% of castration-resistant prostate cancer (CRPC) cases can be attributed to androgen receptor amplification [9, 10]. Antiandrogens can lose their AR antagonism and behave as partial agonists in response to AR overexpression [11]. Although AR mutations do not seem prevalent in the early stages of prostate cancer, scientists have documented different mutations upon relapse during antiandrogen therapy [12]. Differences have been observed in the reports describing AR mutation incidents. Recently, gene sequencing studies confirmed that mutations occur in 20% of patients with metastatic CRPC [13]. 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 [14]. The clinical symptoms 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 [15].

A recent study has shown a particular expression of AR splice variants with different C-terminal extensions encoded by cryptic exons from the intron regions between canonical coding exons in castration-resistance [16]. These variants often occur in the LBD and may exhibit constitutive AR activation. Although reports have shown that these variants still require full-length AR to function, recent studies have suggested that the expression of these variants is sufficient to elicit transcription of AR target genes in the absence of androgen and confers resistance against the novel antiandrogen enzalutamide [17, 18].

As mentioned above, prostate cancer cells continually undergo AR mutations that switch the antiandrogen role from antagonist to agonist. Eventually, a relapse leading to lethal CRPC is observed. Several rational antiandrogen design strategies have been developed to target the mutant AR. Such strategies typically focus on AR-HBP [19]. However, targeting the AR is an ongoing effort and remains a major challenge.

The search for potent antitumor agents has recently involved the examination of both natural and synthetic steroidal glycosides as they exhibit antitumor activities in different tumor cell lines [20–24]. One example of natural antitumor agents is saponin OSW-1, which exhibits relatively low toxicity against healthy cells but inhibits the growth of different cancer cells [23–25]. The synthesis of OSW-1 and its analogs has been studied extensively [21–24]. In 2004, Shi and coworkers successfully synthesized one of the OSW-1 analogs (SBF-1), which demonstrated a more potent inhibitory effect against cancer cells than OSW-1 [24]. The mechanisms of SBF-1 in different types of cancers have been elucidated [26–28].

In this study, we reported a novel anti-androgen mechanism of SBF-1. It exhibits potent cytotoxicity towards two prostate cancer cell lines, LNCaP and PC3/AR$^+$ cells, with considerably low IC_{50} values. This compound strongly attenuates IGF-1/AKT/FOXO1/PCNA signaling, promotes apoptosis and cell cycle arrest. Interestingly, SBF-1 showed significant binding to AR-DBD, thereby blocking the interaction between AR and its target genes. Hence, SBF-1 is different from the compounds blocking androgen-AR interactions and could be a potential drug for treating advanced prostate cancer cases.

Materials and methods

Reagents

SBF-1 is a steroidal glycoside provided by co-author Prof. Biao Yu. Its structure is shown in Fig. 1a. Primary antibodies (AR, rabbit monoclonal, Cat# 5153 T, 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 antibodies (phosphorylated $AR_{S515,}$ rabbit polyclonal, Cat# ab128250, RRID: AB_11141430) were purchased from Abcam (China). Primary antibodies (IGF-1, rabbit polyclonal, Cat# PA1–86913, RRID: AB_2122127) were purchased from Thermo Scientific. Primary antibodies (β-actin, mouse monoclonal, Cat# sc-47,778 HRP, RRID: AB_2714189), (GAPDH mouse monoclonal, Cat# sc-32,233, RRID: AB_627679) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sunshine Biotechnology (Nanjing, China). 5α-Dihydrotestosterone (DHT) solution was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the plasmids: pcDNA3.1 [Flag-AR_{WT}, Flag-$AR_{\Delta DBD,}$ 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, USA).

Cell culture

LNCaP cells expressing AR with a novel mutation T878A in the AR ligand-binding domain, PC3 cells (AR-negative), PC3/AR$^+$ cells, a stable AR_{WT} expressing cell line initially obtained as a sub-line from the parental cells PC3, and human

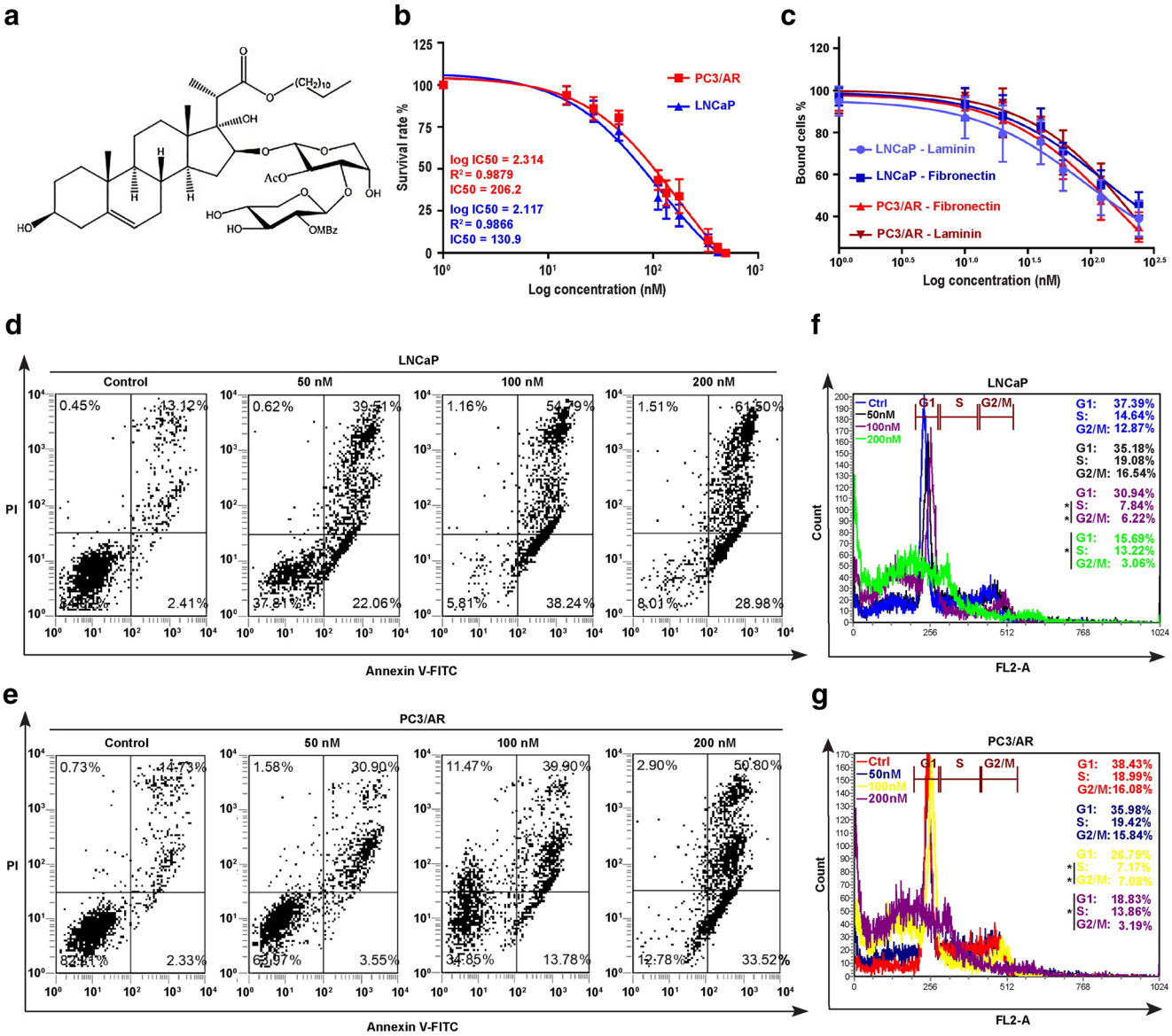

Fig. 1 SBF-1 exhibited potent cytotoxic effects against LNCaP and PC3/AR$^+$ Prostate cancer cells. **a** Chemical structure of SBF-1. **b** 1×10^5 LNCaP or PC3/AR$^+$ cells were seeded into 96-well microplates and incubated with various concentrations of SBF-1 for 24 h. Cell viability was determined by MTT assay. **c** Cell adhesive ability was tested toward fibronectin and laminin. **d** and **e** Annexin V/PI staining determined the percentages of apoptotic cells. **f** and **g** The cell cycle was determined by PI staining. Values in B and C were shown as the mean ± SEM. Data in D-G were representative of three independent experiments

embryonic kidney cell line HEK293T were obtained from the Chinese Academy of Medical Sciences (Tianjin, China). All cells were maintained in Dulbecco's Modified Eagle's medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 U/mL penicillin, and 100 mg/mL streptomycin and incubated at 37° in an incubator containing 5% CO2.

MTT assay

Cells (1×10^3) were seeded into 96-well plates and incubated with various concentrations of SBF-1 for the indicated periods. The survival rate was determined as described previously [26].

Apoptosis, cell cycle, and cell adhesion assay

Apoptosis in cells was determined by annexin V/PI staining. Briefly, the cells were evaluated using flow cytometry after the addition of FITC-conjugated annexin V and PI, as previously described [29]. Annexin V$^+$ / PI$^-$ and annexin V$^+$ / PI$^+$ cells were considered apoptotic cells in the early and late phases, respectively. Samples were analyzed by flow cytometry using the FACScan (Becton Dickinson).

For cell cycle analysis, the cells were stained with propidium iodide (PI) as previously described [30], and the cell cycle distribution was analyzed by flow cytometry using the FACScan (Becton Dickinson). The percentages of cells in the G0/G1, S, and G2 phases were counted and compared.

Cell adhesion assay was performed as reported previously [26].

Western blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer, as described previously [28]. 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 reverse transcribed with Oligo (dT) primers (Takara, Tokyo, Japan). The cDNA products were subjected to RT-PCR. cDNA amplification was performed for 35 cycles (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) using *Taq* DNA polymerase (Promega, Shanghai, China). The RT-PCR products were electrophoresed on a 2% agarose gel and visualized using 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. β-actin was used as the loading control. The primer sequences used in this study are listed 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 six wells plates to reach a confluence of 1×10^6 cells and then transfected with the siRNA using Lipofectamine RNAi MAX (Thermo Scientific) according to the manufacturer's protocol. The following sequences were used:

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 the AR_{WT} expression plasmid. Cell extract (5 μg) was collected 36 h after transfection. It was incubated with γ32P ATP-labeled oligonucleotide probes with or without SBF-1 along with the consensus AR-binding sites ARE-1 (Santa Cruz Biotechnology) in a buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, two mM $MgCl_2$, two mM spermidine, 0.5 mM dithiothreitol, one μg dI-dC, and 10% glycerol for 20 min at room temperature. This was followed by the resolution of complexes on 7% native PAGE. For super-shift analysis, we used an antibody against AR (Cell Signaling Technologies) [31].

The dissociation constants of the protein-DNA complexes were evaluated 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 by dividing the volume of the band corresponding to the bound DNA by the sum of the bands corresponding to free and bound DNA. Data were fitted to a modified two-state binding equation to determine the apparent dissociation constants for each protein-DNA complex as reported previously [32].

Androgen receptor competitor assay

PC3 cells were transfected with the flag-AR_{WT} construct, and the AR-protein was purified from the total protein extract along with the removal of flag tag using the enterokinase enzyme as reported previously [33]. We used the PolarScreen™ Androgen Receptor Competitor Assay Kit

(Green; cat # A15880, Thermo Fisher) to check the possible binding of SBF-1 to the AR ligand-binding domain (AR-LBD) following the manufacturer's protocol.

Microscale thermophoresis (MST)

GFP-AR constructs were purchased from Gene-Script (China). Each construct was transfected in PC3 cells. The total protein was extracted in the MST buffer, followed by the addition of SBF-1 in a serial dilution of 16 folds. The assay was performed as previously reported [34], and the binding affinity was examined using Monolith NT.115.

Isothermal titration calorimetry (ITC)

Flag-AR_{WT} and Flag-$AR_{\Delta DBD}$ were expressed and purified following a previously published protocol [35], followed by removal of the Flag tag, as previously described [33]. The binding affinity between SBF-1 and both isoforms was examined using MicroCal ITC 200 [36].

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 DNA pull-down assay was performed as described in Fig. S3A.

WT AR and its mutant isoforms were synthesized and subcloned into a pGL3 vector (Promega, Shanghai, China). PC3 cells were transfected with AR constructs (WT and mutants). PC3 cells were further treated with SBF-1 (200 nM), DHT (10 nM), or a combination of SBF-1 and DHT. Luciferase activity was determined 6 h after transfection.

Animal models and drug administration

Male BALB/c nude mice (6–8 weeks old) were obtained from Jiangsu Gempharmatech Co. Ltd. (Nanjing, China). The mice were kept under pathogen-free conditions in type IV Makrolon cages (six mice per cage) with an airflow cabinet at 23 °C, 12 h/12 h day/night cycle. Sterilized food and sterilized acidified water were regularly provided to the animals. 1×10^6 LNCaP and PC3/AR^+ cells were injected subcutaneously into the right flank. After 12 days of injection, all mice demonstrated tumor formation, which was marked as day 0. Vehicle (0.05% DMSO in PBS), 10, and 30 μg/kg SBF-1 were intraperitoneally injected into animals belonging to each group every day for 14 days. We measured the body weights and tumor volume simultaneously. For the tumor growth assay, mice were euthanized 14 days after drug treatment, and the weight of the tumor was measured. All animal welfare and experimental procedures were strictly performed following 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. We made all efforts to minimize animal suffering and to reduce the number of animals to be used for the study.

In Silico analysis

We used Autodock vina 4.2 for the docking analysis of SBF-1 to AR-DBD (PDB ID: 1R4I). Of the many docking poses, only those with the highest docking score were selected. The best binding affinity score was [−11.3 kcal/mol]. During the analysis of the predicted binding affinity, a high absolute value of the energy indicated a high affinity of the corresponding ligand and receptor, as this datum represents the free energy of binding in AutoDock Vina v4.2 docking software.

Statistical analysis

Data are expressed as mean ± SEM. We used the Student's *t* test to evaluate differences between groups. Values with $P < 0.05$ were considered significant. All in vitro data were obtained from at least three independent experiments.

Results

SBF-1 demonstrated potent cytotoxicity against LNCaP and PC3/AR+ prostate cancer cells

We incubated LNCaP and PC3/AR^+ with different concentrations of SBF-1 for 3 h. SBF-1 demonstrated potent cytotoxicity toward LNCaP and PC3/AR^+ cells and suppressed their growth (Fig. 1b).

The treated LNCaP and PC3/AR^+ cells were analyzed for adhesiveness to fibronectin and laminin. SBF-1 significantly inhibited the adhesion of LNCaP and PC3/AR^+ cells to fibronectin and laminin in a concentration-dependent manner (Fig. 1c).

Next, we performed apoptosis and cell cycle analysis. Compared to the control cells, the percentage of apoptotic cells significantly increased, and cell cycle analysis showed G1 and G2/M phase arrest in SBF-1-treated LNCaP and PC3/AR^+ cells (Fig. 1d-g).

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

Considering the vital role of AR signaling in prostate cancer cell growth, we next examined the effect of SBF-1 on AR/IGF-1 and IGF-1/AKT/FOXO1/PCNA signaling pathways. As shown in Fig. 2a, SBF-1 downregulated the protein expression of IGF-1, PCNA, Bcl-2, pAR_{S515}, $pAkt_{S473}$, and $pFOXO1_{S256}$ but had no effect on the total protein expression of AR, AKT1, and FOXO1 in LNCaP

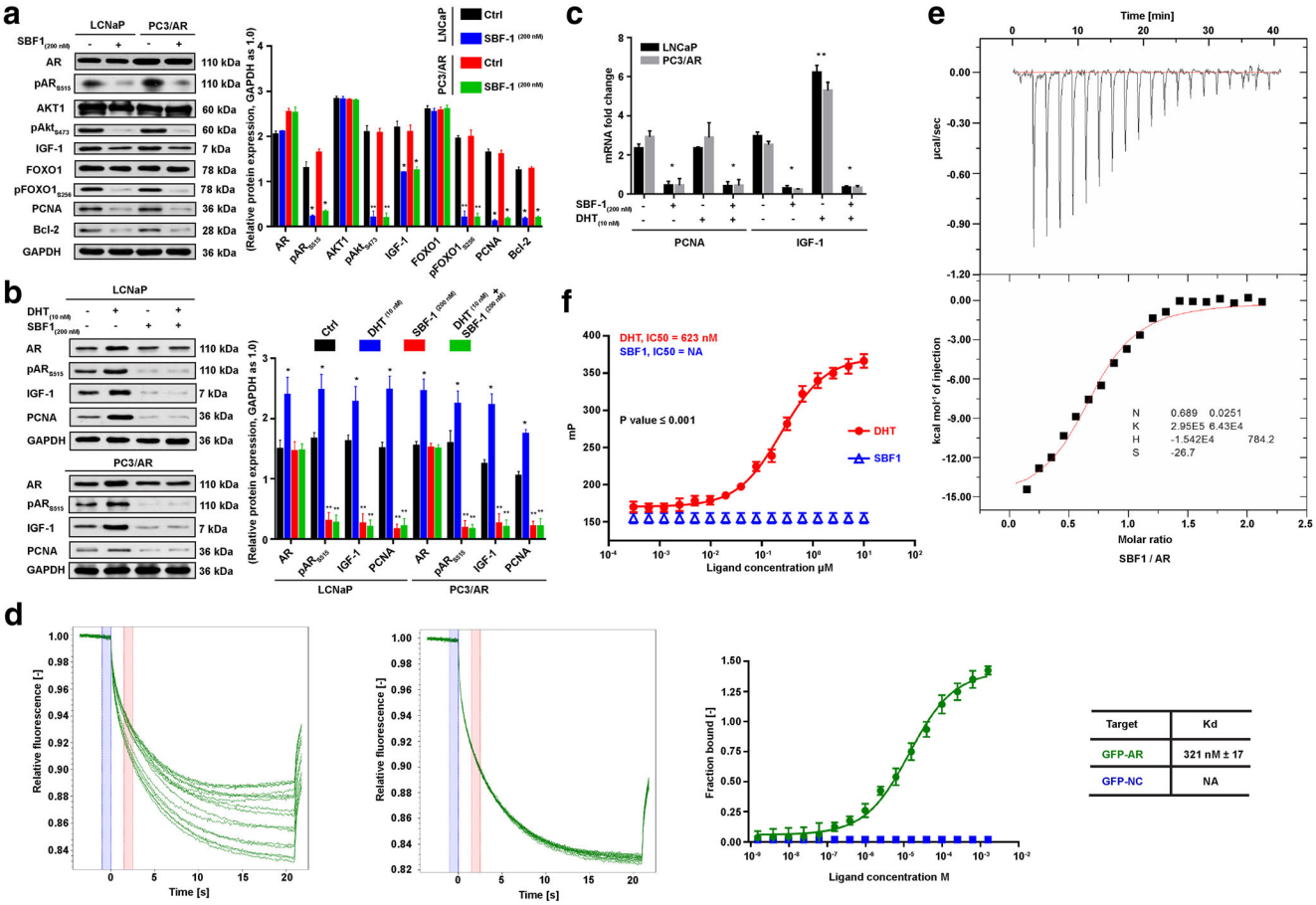

Fig. 2 SBF-1 bound to the AR and inhibited its downstream signaling. **a**, **b** LNCaP and PC3/AR$^+$ cells were treated with SBF-1 for 6 h at 200 nM final concentration in the presence or absence of DHT (10 nM). The protein levels of AR, pAR$_{S515}$, p-AKT$_{S473}$, AKT1, IGF-1, FOXO1, p-FOXO1$_{S256}$, PCNA, and Bcl-2 were determined in the whole lysate by western blot. GAPDH was used as a loading control. **c** LNCaP and PC3/AR$^+$ cells were treated in the presence or absence of SBF-1 (200 nM) or DHT (10 nM) for 6 h, and the mRNA levels of IGF-1 and PCNA were determined. **d** Cell lysate from GFP-AR$_{WT}$ transfected PC3 cells was incubated with 16 different doses of SBF-1 (serial dilution), and the binding affinity was determined as the change in the GFP fluorescence using MST assay. **e** Purified AR$_{WT}$ at a final concentration of 200 µM was used in the sample cells. One µl injection (19 injections in total) of 2 mM SBF-1 was titrated into the sample with 120 s spacing between each injection and a stirring speed of 1000 rpm. The ligand background was obtained by titrating micromolar amounts of SBF-1 into the buffer. The data from the ligand were averaged and subtracted from the SBF-1 into AR$_{WT}$ ITC data. Origin 7.0 (OriginLab) was used to analyze the binding isotherms. **f** Purified AR$_{WT}$ was incubated with SBF-1 and DHT along with the substrate Fluormone AL Green and the fluorescent shift was determined. Values in C were shown as the mean ± SEM of three experiments. $^{**}P < 0.01$. Data in A, B, and D-F were representative of three independent experiments

and PC3/AR$^+$ cells. This result suggested that SBF-1 directly targeted and downregulated the expression of components involved in the AR/IGF-1 and IGF-1/AKT/FOXO1/PCNA pathways. We assumed that SBF-1 might have focused upstream of AR signaling. To further confirm the effect of SBF-1 on AR signaling, we used DHT to stimulate LNCaP and PC3/AR$^+$ cells and activate AR signaling. DHT stimulation significantly increased the AR protein expression and its phosphorylation, it also increased IGF-1 and PCNA protein expression levels.

In contrast, SBF-1 significantly blocked the protein expression of AR (increased by DHT), pAR$_{S515}$, IGF-1, and PCNA in LNCaP and PC3/AR+ cells (Fig. 2b). This result indicated a dual effect of SBF-1 on the AR/IGF-1 axis and downstream signaling. In addition, as shown in Fig. 2c, SBF-1 significantly suppressed the mRNA expression of IGF-1 and PCNA in the presence or absence of DHT. These findings suggested that SBF-1 could 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 the AR since it is a steroidal glycoside. We first conducted an MST analysis to examine whether there is binding between SBF-1 and AR. We transfected HEK293T cells with GFP-AR$_{WT}$, and the total cell lysate was extracted and incubated with different doses of SBF-1 to determine the binding affinity between SBF-1 and the AR. As shown in Fig. 2d, the binding affinity between SBF-1 and the AR

was 321 nM, indicating a sturdy binding. Furthermore, we used the ITC technique to confirm the binding between SBF-1 and AR. The calculated ΔK of SBF-1 binding to the purified AR_{WT} protein was 2.95× 10–5 M, confirming the binding between SBF-1 and the AR (Fig. 2e).

We then performed the androgen receptor competitor assay to check whether SBF-1 targeted the AR-LBD. We transfected HEK293T cells with Flag-$AR_{WT,}$ and the AR_{WT} flag-tag was cleaved. Subsequently, the untagged AR protein was incubated with a fluorescent substrate in the presence of SBF-1 or DHT, as mentioned in the methods section. The measured fluorescence signal indicated a significant shift in the fluorescence polarization, revealing a competitive substrate binding to the AR-LBD by the DHT. This result confirmed the binding of DHT to the AR-LBD. However, SBF-1 did not cause any signal shift, indicating that the AR-LBD is not the binding site of SBF-1 (Fig. 2f). The aforementioned findings suggest that SBF-1 targeted the AR at a different binding site than AR-LBD.

SBF-1 bonded with the AR mutants and inhibited their activation

AR mutations are involved in castration-resistant prostate cancer (CRPC). The most frequently identified AR point mutations include T878A [37–40], W742C [41–47], L702H [43–45, 47] and F876L mutations [48]. Based on these findings, we constructed AR plasmids that possess these frequent mutations to examine the effect of SBF-1 on mutant AR (Fig. 3). The constructs were tagged with GFP and transfected into PC3 cells. After 36 h of transfection, we collected the cell lysate to examine the binding between SBF-1 and the AR. We used the MST assay to detect the binding affinity between SBF-1 and the AR mutants. SBF-1 showed a strong binding affinity toward L702H (10 μM), W742C (610 nM), F876L (1.9 μM), and T878A (385 nM) (Fig. 3a). We also examined the activity of each AR mutant compared to the wild type using a reporter gene assay. The AR-transfected PC3 cells were treated with SBF-1, DHT, or both. The DHT stimulation of AR-WT and mutant (0.1 and 10 nM) cells increased the luciferase activity, whereas SBF-1 demonstrated no changes in luciferase activity in both WT and mutants. Surprisingly, the combined treatment of DHT and SBF-1 did not cause any change in the luciferase activity, suggesting that SBF-1 might bind to the AR blocking its activity by DHT (Fig. 3b). We further examined the effect of SBF-1 on AR phosphorylation. We treated the GFP-AR-transfected PC3 cells with DHT or a combination of SBF-1 and DHT for an additional six h. SBF-1 significantly reduced the phosphorylation of the AR_{WT} and its mutants regardless the presence of DHT (Fig. 3c).

SBF-1 bonded to the AR-DBD, blocking the interaction between the androgen receptor and its target genes

The aforementioned results indicate the presence of an alternative SBF-1 binding site in the AR than in the LBD. We constructed an AR consensus recognition sequence ARE-1 to explore whether SBF-1 affected AR-DBD [6]. Using the EMSA technique, we incubated the constructed ARE-1 sequence with the purified AR_{WT} protein with or without SBF-1 and determined their interaction. EMSA analysis demonstrated a shifted band that appeared in the absence of SBF-1 at a Kd value of 370 nM. However, no shifted band appeared in the presence of SBF-1 (Fig. 4a), suggesting that the AR-DNA interaction was stymied by SBF-1. We then constructed $AR_{\Delta DBD}$ (lacking the DNA binding domain) and analyzed the binding affinity between SBF-1 and $AR_{\Delta DBD}$ using MST assay. The MST results demonstrated a tenuous binding between SBF-1 and the $AR_{\Delta DBD,}$ with a value of 698 μM (Fig. 4b).

Furthermore, we expressed and purified the $AR_{\Delta DBD,}$ followed by employing the ITC technique to determine the thermodynamic parameters of the interaction between SBF-1 and the $AR_{\Delta DBD}$. The result indicated no binding between SBF-1 and the $AR_{\Delta DBD}$ (Fig. 4c). We further used the ChIP-qPCR assay to analyze the effect of SBF-1 on IGF-1 enrichment status. As shown in Fig. 4e, DHT stimulation induced a significant increase in IGF-1 enrichment, whereas SBF-1 inhibited this enrichment. SBF-1 also inhibited the PCNA gene expression. In silico analysis supported the possible binding between SBF-1 and the AR-DBD, and the DNA pull-down assay confirmed SBF-1 mediated blockade of the AR-binding to its target gene IGF-1 (Fig. S2 and S3). These findings were in accordance with our previous results.

SBF-1 inhibited the activation of the AR and its subsequent signaling

Glucose stimulation significantly activates IGF-1 followed by subsequent activation of the AKT-FOXO1 axis [49, 50]. We treated LNCaP and PC3/AR+ cells with 20 mM glucose, 200 nM SBF-1, and 5 nM DHT or both SBF-1 and DHT for three h. SBF-1 decreased the protein levels of IGF-1, PCNA, $pAKT_{S473,}$ and pFOXO1 regardless of the presence of glucose or DHT (Fig. 5a and b), whereas DHT and glucose stimulation increased IGF-1 mRNA expression levels (Fig. 5c). In Fig. 5d and e, we knocked down IGF-1 and FOXO1, respectively, in LNCaP and PC3/AR^{+} cells. SBF-1 and DHT demonstrated an opposite effect on the AR downstream signal proteins PCNA and Bcl-2. In the cells treated with a combination of DHT and SBF-1, SBF-1 significantly blocked the expression of PCNA and Bcl-2 that was increased by DHT. These results suggested that SBF-1 might demonstrate anti-prostate cancer activity

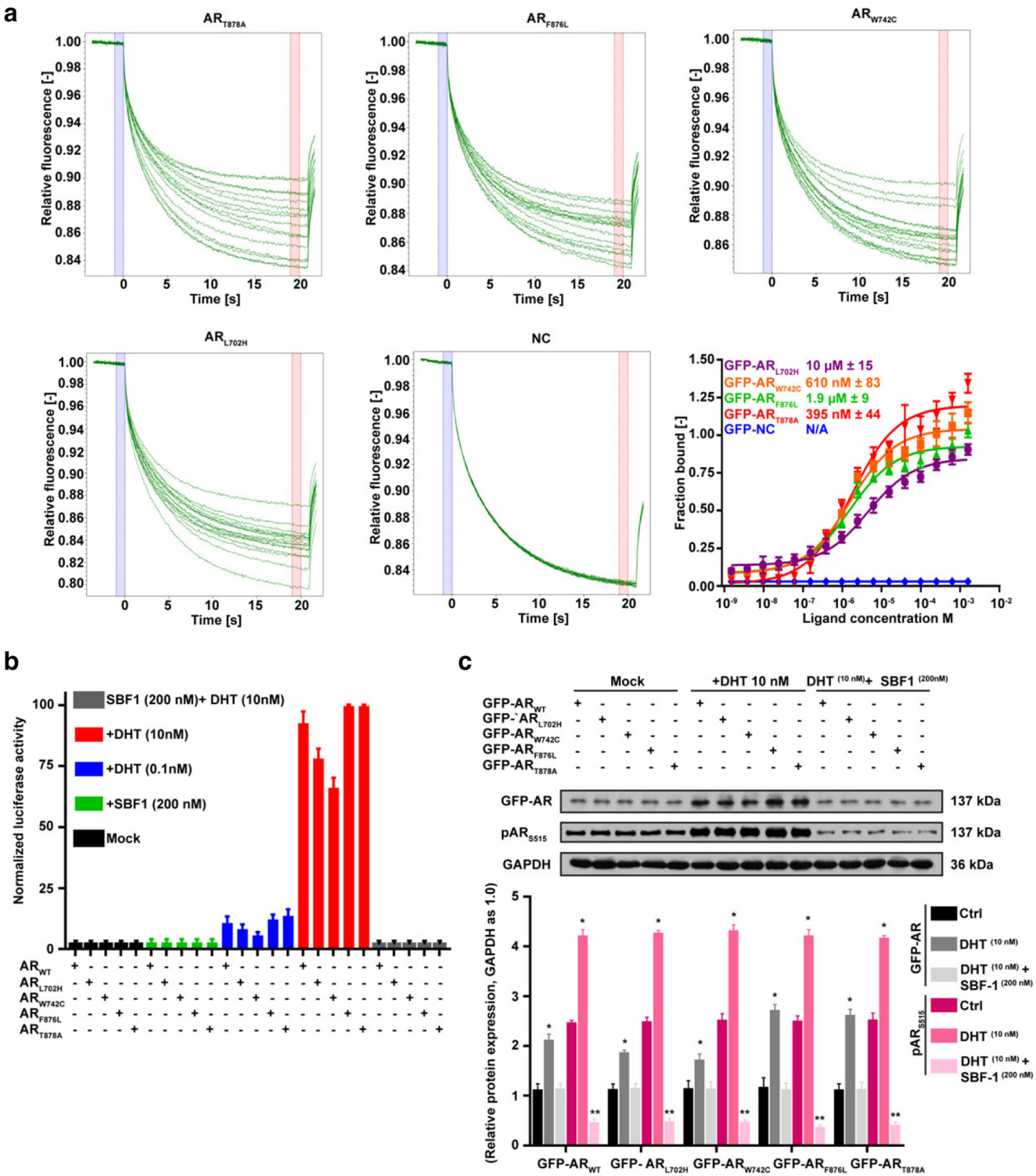

Fig. 3 SBF-1 inhibited the AR activity. **a** AR_{WT} and its mutants AR_{L702H}, AR_{W742C}, AR_{F876L}, and AR_{T878A} were tagged with GFP and transfected into PC3 cells. The total lysate was collected and incubated with different doses of SBF-1, and the binding affinity was determined as the change in the GFP fluorescence using MST assay. **b** AR_{WT} and its mutants AR_{L702H}, AR_{W742C}, AR_{F876L}, and AR_{T878A} reporter systems were treated with either SBF-1 or DHT, and the transcription activity was measured. **c** AR_{WT} and its mutants AR_{L702H}, AR_{W742C}, AR_{F876L}, and AR_{T878A} was transfected into PC3 cells, and the total lysate was used to determine the protein levels of AR and pAR_{S515} by western blot. Values in A and B were shown as the mean ± SEM. Data in A and C were representative of three independent experiments

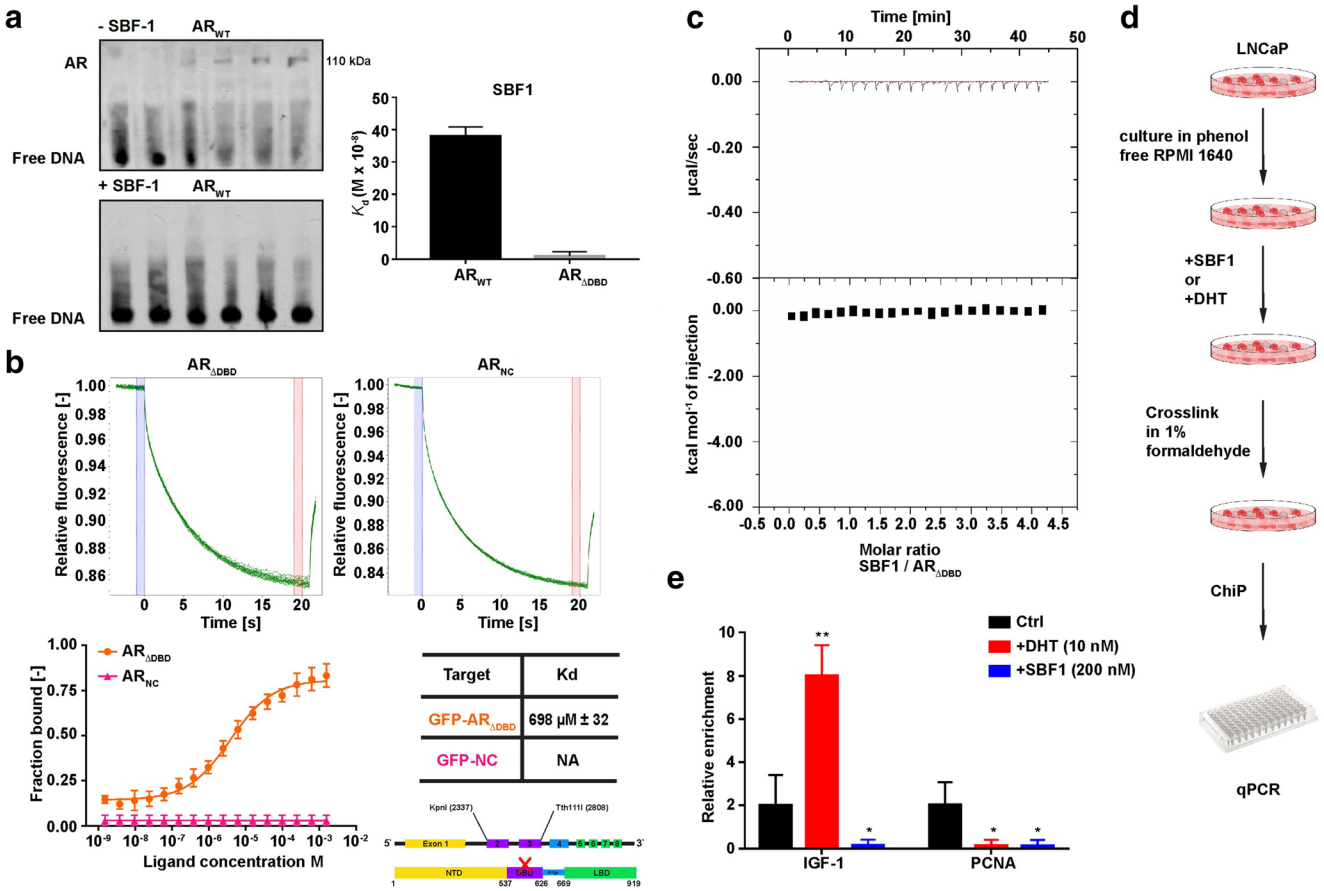

Fig. 4 SBF-1 is bound to AR-DBD, blocking the AR from binding to its target genes. **a** EMSA was performed to determine the SBF-1 effect on the binding between the AR_{WT} and the ARE-1 fragment. The binding Kd was determined as described in the methods. **b** Cell lysate from GFP-$AR_{\Delta DBD}$-transfected PC3 cells was incubated with different doses of SBF-1, and the binding affinity was determined as the change in the GFP fluorescent using MST assay. The $AR_{\Delta DBD}$ editing is shown in the illustrative graph. **c** Purified $AR_{\Delta DBD}$ at a final concentration of 200 μM was used in the sample cells. One μl injection (19 injections in total) of 2 mM SBF-1 was titrated into the sample with 120 s spacing between each injection and a stirring speed of 1000 rpm. The ligand background was obtained by titrating micromolar amounts of SBF-1 into the buffer. The data from the ligand were averaged and subtracted from the SBF-1 into $AR_{\Delta DBD}$ ITC data. Origin 7.0 (OriginLab) was used to analyze binding isotherms. **d** Schematic graph of Chip experiment. (E) ChiP-PCR analysis of AR_{WT} from PC3/AR^+ cells was performed according to the ChiP kit manufacture protocol. ChiP primers were listed in the methods. Values in E were shown as the mean ± SEM of three experiments. $^*P < 0.05$, $^{**}P < 0.01$. Data in A-C were representative of three independent experiments

despite the endogenous levels of androgens and the AR mutations.

SBF-1 inhibited the tumor growth in nude mice bearing either LNCaP or PC3/AR+ xenografts

Following our previous findings, we analyzed the inhibitory effect of SBF-1 in vivo. Balb/C nude mice were injected subcutaneously with LNCaP or PC3/AR^+ cells to establish prostate cancer xenograft models. After the tumor size reached 50 mm^3, we intraperitoneally administered 10 and 30 μg/kg of SBF-1. SBF-1 significantly reduced the tumor size of the animals in a dose-dependent manner (Fig. 6a and b). We injected the experimental animals with ICG dye-IGF-1 conjugate to determine the tumor size and progress. Three hours post-injection, we observed that SBF-1 remarkably reduced the IGF-1 fluorescence intensity in a dose-dependent manner, suggesting an association between potent tumor growth inhibition and reduced IGF-1 expression (Fig. 6c and d). SBF-1 significantly decreased tumor size in a dose-dependent manner (Fig. 6e and f). Besides, SBF-1 inhibited the protein expression of pAR_{S515}, $pAKT_{S473}$, $pFOXO1_{S256}$, IGF-1, PCNA, and Bcl-2 in the tumor cells (Fig. 6g and h) along with the expression of IGF-1 and PCNA genes in both xenograft models in a dose-dependent manner (Fig. 6i).

Discussion

The present study demonstrated inhibition of the binding of AR to its target gene, i.e., IGF-1, by a novel antiandrogen SBF-1, thereby inducing apoptosis and cell cycle arrest. Comparing the effect of SBF-1 on androgen-independent prostate cancer cells (PC3) and androgen-dependent (LNCaP

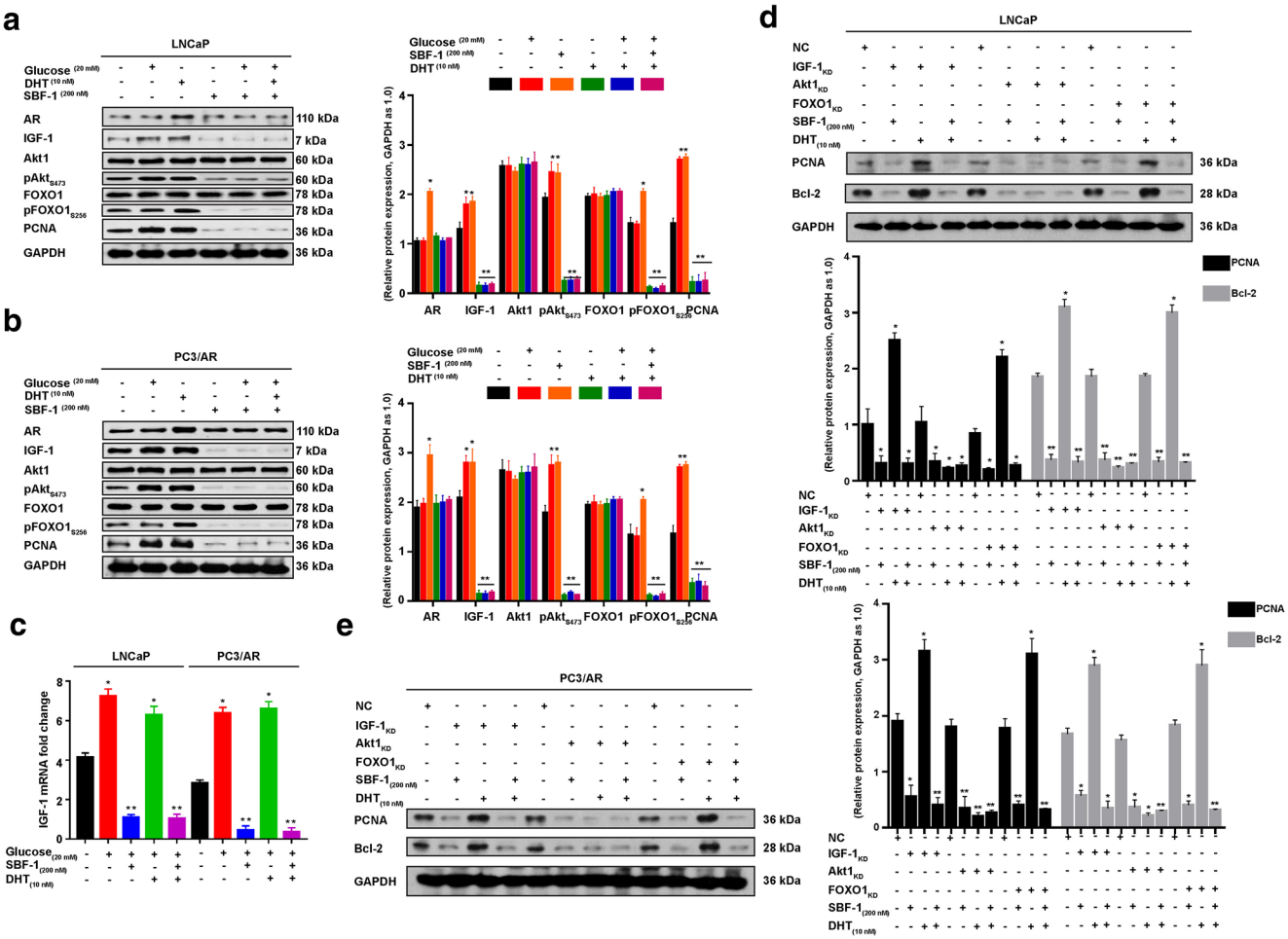

Fig. 5 SBF-1 suppressed the AR activation and its subsequent signaling in LNCaP and PC3/AR$^+$ cells. LNCaP and PC3/AR$^+$ cells were treated in the presence or absence of SBF-1200 nM, DHT 5 nM, and Glucose 10 mM. **a**, **b** The protein levels of AR, IGF-1, AKT1, p-AKT$_{S473}$, FOXO1, p-FOXO1$_{S256}$, and PCNA were determined by western blot. GAPDH was used as a loading control. **c** Q-PCR determined the mRNA expression of IGF-1. **d**, **e** IGF-1, AKT1, or FOXO1 was silenced independently in LNCaP or PC3/AR$^+$ cells. The effect of SBF-1 on DHT-induced PCNA and Bcl-2 expressions was determined by western blot. Values in C were shown as the mean ± SEM of 3 experiments. $^{**}P < 0.01$. Other data were representative of three independent experiments

and PC3/AR+) cells showed that SBF-1 might have different inhibitory mechanisms in different androgen dependent cells. The difficulty of associating the distinct inhibitory effect of SBF-1 in each case led us to believe that SBF-1 is a potential treatment for prostate cancer. However, focusing on androgen-dependent cases is feasible as it is considered the central issue in prostate cancer treatment.

AR is a hormone-inducible transcription factor that drives tumor-promoting gene expression and represents an important therapeutic target in prostate cancer [51]. Prostate cancer patients with higher AR expression demonstrate a lower overall survival rate (Fig. S4). Currently, small molecules used in prostate cancer treatment mainly interfere with steroid recruitment to prevent AR-driven tumor growth [52, 53]. However, such small molecules are rendered ineffective in advanced prostate cancer by the emergence of LBD mutations or expression of constitutively active variants such as AR-V7 that lack the LBD. In our investigations, we utilized two types of prostate cancer cells, namely, LNCaP and PC3/AR$^+$ cells. Prostate cancer cell line LNCaP expressed AR with a novel mutation T878A in AR-LBD, similar to human prostatic adenocarcinoma. PC3/AR$^+$ cells demonstrated a stable expression of AR$_{WT}$, originally derived as a sub-line from the parental AR-negative PC3 cells. Using these two cell lines, we aimed to find a novel inhibitor targeting the AR in prostate cancer cells harboring either AR$_{WT}$ or LBD mutant-AR.

Subjecting LNCaP and PC3/AR$^+$ prostate cancer cell lines to SBF-1 treatment in multiple assays revealed that it significantly inhibited their adhesion to fibronectin and laminin, proliferation, increased the percentage of apoptotic cells, and caused cell cycle arrest in G1 and G2/M phases (Fig. 1b-g). We also analyzed the inhibitory effect of SBF-1 on PC3 cells (Androgen receptor-negative cells). Surprisingly, SBF-1 inhibited the adhesive ability of PC3 cells against fibronectin and laminin and inhibited its proliferation. Besides, SBF-1 promoted cell apoptosis in PC3 cells; however, no cell cycle

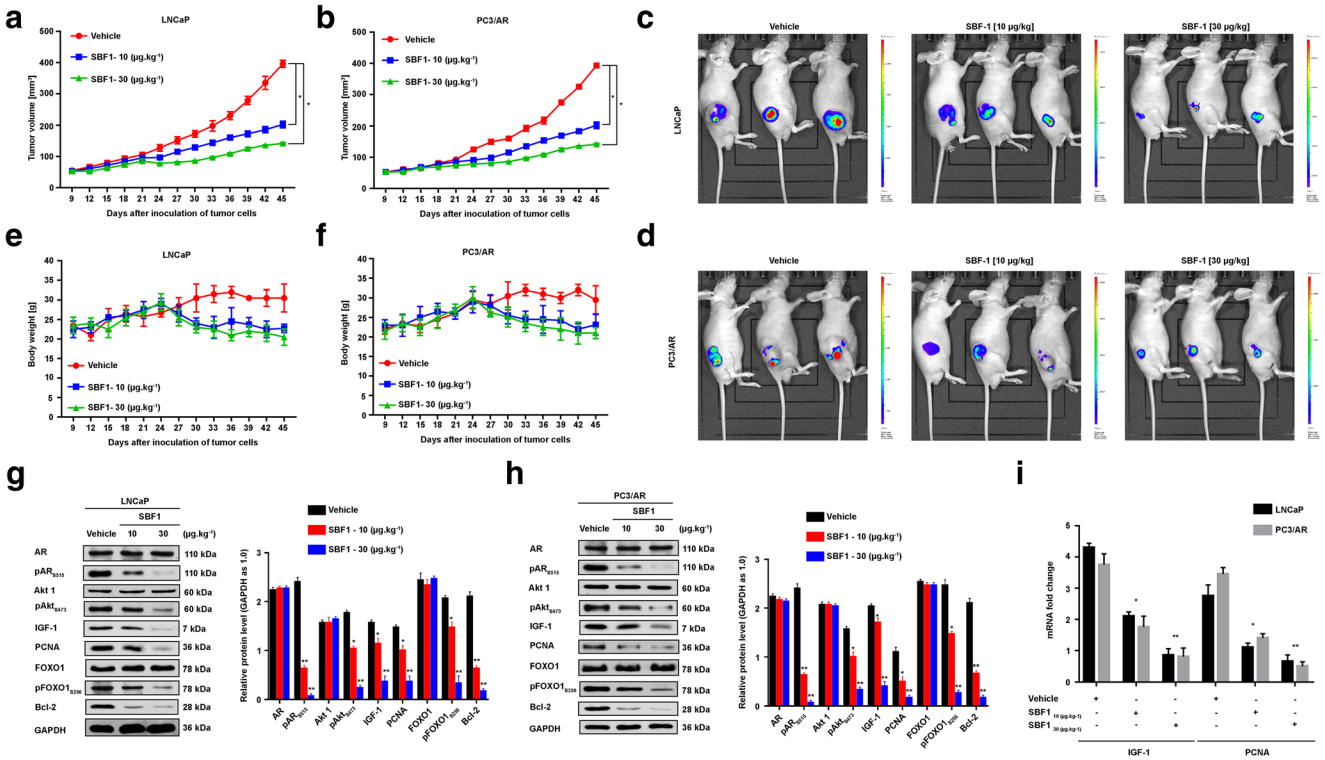

Fig. 6 SBF-1 inhibited prostate cancer tumor growth in vivo. **a**, **b** Tumor growth of LNCaP and PC3/AR$^+$ cells in mice after treatment of 10 and 30 μg/kg of SBF-1 for 45 days. **c**, **d** Tumor size progression in both LNCaP and PC3/AR$^+$ models, indicated by IGF-1 levels in tumor tissue by ICG-IGF-1 conjugated dye in mice. **e**, **f** Body weights of tumor-bearing mice during the treatment of SBF-1. **g**, **h** Effect of SBF-1 on protein expression of various members in AR/IGF-1 and IGF-1-AKT-FOXO1/PCNA signaling in the tumor tissues of LNCaP or PC3/AR$^+$ cancer-bearing mice. **i** mRNA levels of IGF-1 and PCNA from each treated mice group after treatment with SBF-1 at 10 and 30 μg/kg. Values in A and B were shown as the mean ± SEM of 6 mice. $^*P < 0.05$, $^{**}P < 0.01$, vs Vehicle. Data in C, D, G, H, and I, were representative of six mice or three independent experiments

arrest was observed (Fig. S1A, B, F, and G). This difference in the inhibitory effects indicates that SBF-1 is a potent anti-prostate cancer agent. However, we focused our investigations on androgen-dependent prostate cancer represented by LNCaP and PC3/AR$^+$ cells.

Endogenous androgens, including testosterone and dihydrotestosterone, activate the AR [54]. AR mediates the growth of benign and malignant prostate cells in response to DHT. In prostate cancer patients undergoing androgen deprivation therapy, AR drives prostate cancer growth despite low circulating levels of testicular androgen and normal adrenal androgen levels [55]. Usually, prostate cancer cells gradually lose their dependence on AR and become resistant to hormonal therapy. Various hormonal manipulations in castrate-resistant prostate cancers proved ineffective as the cancer cells maintain the AR expression [56–60]. IGF-1 is a transcription product of the AR; it can independently activate the AR in the absence of DHT with a mechanism that involves downstream phosphorylation of either the AR or its associated proteins [61, 62]. Previous reports indicated that the inhibition of IGF-1 could suppress prostate cancer cell growth [63].

In contrast, IGF-1 activates downstream proteins, such as AKT kinase, and regulates cell proliferation and survival. AKT is a hormone-activated protein. Additionally, growth factors and various drugs are involved in AKT activation [63–65]. The downstream forkhead transcription factor family FOXO plays a vital function in cell apoptosis and survival in various cell types, which can be phosphorylated by AKT kinase [66]. Based on these findings, we analyzed the effect of SBF-1 on AR signaling with DHT stimulation. As shown in Fig. 2a, SBF-1 significantly decreased the protein expression of IGF-1, PCNA, Bcl-2, pAR_{S515}, $pAKT_{S473}$, and $pFOXO1_{S256}$, without affecting the expression of AR, AKT1, and FOXO1 in LNCaP and PC3/AR$^+$. In PC3 cells, SBF-1 inhibited the expression of PCNA, Bcl-2, pAR_{S515}, $pAKT_{S473}$, and $pFOXO1_{S256}$ but had no effect on IGF-1 expression (Fig. S1C). This result suggests that SBF-1 might have different inhibitory mechanisms in diverse AR-dependent cases.

DHT stimulation significantly increased AR expression and phosphorylation and upregulated the expression of downstream proteins IGF-1 and PCNA. In contrast, SBF-1 downregulated the expression of AR, pAR_{S515}, IGF-1, and PCNA increased upon DHT stimulation in LNCaP and PC3/AR$^+$ cells (Fig. 2b). This result indicated a dual effect of SBF-1 on the AR/IGF-1 axis and its downstream signaling. SBF-1

substantially suppressed the mRNA expression of IGF-1 and PCNA regardless of DHT stimulation (Fig. 2c). In contrast, in PC3 cells, SBF-1 did not affect the IGF-1 mRNA levels but significantly decreased the PCNA gene expression (Fig. S1D). These findings suggest that SBF-1 directly blocked the gene transcription mediated by AR in androgen-dependent cells; however, it showed different effects in androgen-independent cell lines.

We hypothesized that SBF-1 might directly bind to the AR as it is a steroidal glycoside. We used MST and ITC assays to examine the binding between SBF-1 and purified AR_{WT}. The results indicated a strong binding affinity between SBF-1 and the AR_{WT} (Fig. 2d and e). We then performed an androgen receptor competitor assay to examine whether SBF-1 binds to the consensus target, namely, AR-LBD in prostate cancer therapy [67]. However, despite the significant DHT binding to the AR-LBD, SBF-1 did not bind to the AR-LBD (Fig. 2f). The aforementioned findings suggest that SBF-1 targeted different sites in the AR compared to the AR-LBD.

AR point mutations can activate AR signaling in CRPC tumor epithelial cells. Such mutations are rare in untreated prostate cancer but are detected in 15%–20% of CRPC patients [68–70] and up to 40% of CRPC patients treated with AR antagonists [71]. AR point mutations are generally located in the C-terminus LBD; about one-third of such mutations are observed in the transactivation domain NTD [12, 72], resulting in broad ligand specificity. The first and most frequently identified AR point mutation is the flutamide-driven T878A mutation [37–40]. W742C was reported in patients treated with first-generation AR antagonists [41–45, 47]. CRPC patients undergoing abiraterone treatment commonly demonstrate T878A and L702H mutations [43–45, 47]. Moreover, the F876L mutation confers the antagonists-to-agonists that drives phenotypic resistance [48]. Hence, we examined these frequently occurring mutations in CRPC by constructing AR-mutant plasmids and analyzed the inhibitory effect of SBF-1 against such mutations (Fig. 3). SBF-1 demonstrated a robust binding affinity toward all mutant constructs, L702H, W742C, F876L, and T878A (Fig. 3a). It is important to note that SBF-1 inhibited the activity of the AR_{WT} and $AR_{mutants}$ increased by DHT (Fig. 3b). The aforementioned results support the hypothesis that SBF-1 targeted a different site in the AR rather than the LBD.

Accumulating evidence suggests that AR splice variants lacking the LBD coding sequence could promote the growth of castration-resistant prostate cancer; Hence, only NTD and DBD are viable domains targeted by small molecules [16, 17, 73–75]. Inhibition of the splice variant transcriptional activity would be a significant breakthrough in developing new anti-AR drugs [51]. We used the ARE-1 sequence, a consensus recognition site for the AR [6], to determine the effect of SBF-1 on AR-DNA binding. We incubated the constructed ARE-1 sequence and purified AR_{WT} with or without SBF-1. SBF-1 blocked AR_{WT}-binding to ARE-1 (Fig. 4a). Since SBF-1 blocked the AR-DNA interaction, we constructed the $AR_{\Delta DBD}$ (lacking the DNA binding domain). We then performed an MST assay to determine the binding affinity between SBF-1 and the newly constructed $AR_{\Delta DBD;}$ The results showed a weak binding affinity of 689 μM (Fig. 4b). This result suggested that AR-DBD might be a potential target for SBF-1. The results of the ITC technique confirmed that SBF-1 failed to bind to the purified $AR_{\Delta DBD}$ (Fig. 4c). ChIP analysis of the AR-induced gene expression demonstrated that DHT significantly enriched the expression of the AR-target gene IGF-1. However, SBF-1 reduced this enrichment (Fig. 4e). These results suggested that SBF-1 binds to AR-DBD, blocking it from binding to its target genes.

The AR-LBD point mutations are involved in castration resistance. Hence, there is an urgent need for the development of new small molecules capable of the treatment of CRPC via novel mechanisms. To the best of our knowledge, a small molecule, EPI-001, blocked the transactivation of the NTD and specifically inhibited AR without attenuating the transcriptional activities of the steroid receptors [76]. In contrast, targeting AR-DBD is a new strategy for CRPC treatment [51]. However, the development of inhibitors that specifically target the NTD or DBD of the AR has shown little growth [52, 53]. Here, we provided proof of the potent anti-prostate cancer agent, SBF-1, which binds to the AR-DBD, overcoming the occurrence of AR-LBD mutations; this compound efficiently inhibited the growth of LNCaP and $PC3/AR^+$ cells. The IGF-1/AKT/FOXO1 axis is crucial in CRPC. AR stimulation by DHT activates the antiapoptotic IGF-1/AKT/FOXO1/PCNA pathway in LNCaP and $PC3/AR^+$ cells [77]. The activation of IGF-1/AKT/FOXO1/PCNA is critical for cell survival and is involved in CRPC via the modulation of AR expression and its downstream signaling. Here, we used LNCaP cells, which serve as an excellent model to examine advanced-stage prostate cancer with metastatic potential as well as $PC3/AR^+$ cells [78]. These two cell lines provided reliable proof of how SBF-1 treatment affected different prostate cancer cases.

Targeting AR-DBD has recently garnered attention owing to its presence in all AR forms. This will aid in the treatment of castration-resistant prostate cancer [51]. Blocking the AR from regulating its target gene, IGF-1, might be a better strategy for the treatment of castration resistance (Fig. 5). The distinct mechanism of SBF-1 compared to those of the current anti-prostate cancer agents is considered beneficial leading to an improved prostate cancer treatment. Finally, we tested the novel mode of action of SBF-1 in vivo. SBF-1 significantly reduced the tumor size in LNCaP or $PC3/AR^+$ tumor-bearing mice and caused a substantial decrease in the expression of IGF-1 protein and its downstream signaling (Fig. 6).

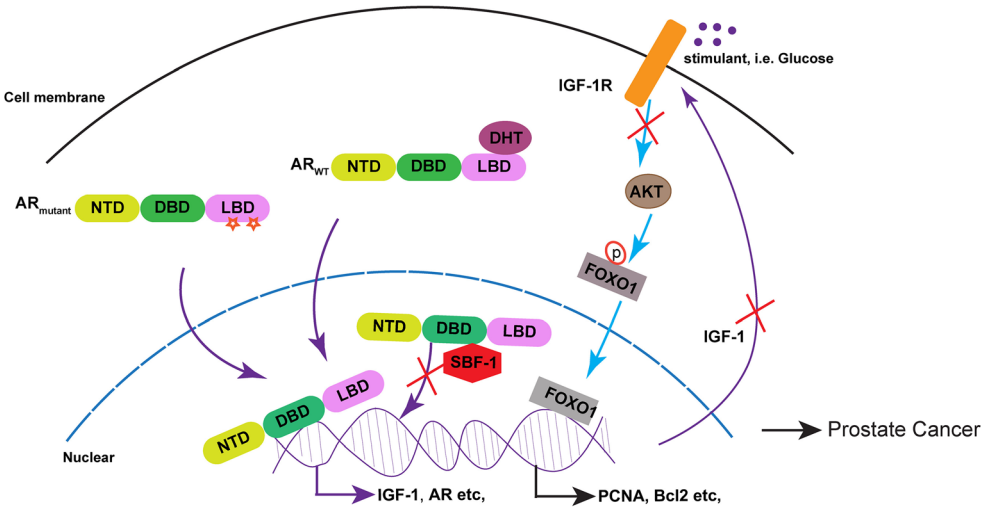

Fig. 7 Illustrative map for the novel action mode of SBF-1. SBF-1 directly binds to AR-DBD and blocks its target genes transcription and the subsequent IGF-1/AKT/FOXO1/PCNA signaling, which overcomes AR's re-activation signaling by mutations in the AR-LBD and thus inhibits tumor growth of advanced prostate cancer

In summary, we present a novel antiandrogen, SBF-1 that targeted AR-DBD and attenuated different variants of the AR (Fig. 7). Our findings demonstrate the potent activity of SBF-1 and its efficient targeting of AR-DBD for the treatment of advanced prostate cancer.

Furthermore, SBF-1 demonstrated a potent cytotoxic effect against AR-negative PC3 cells. Hence, SBF-1 might have different mechanisms for the inhibition of the growth of prostate cancer cells. Thus, it is a promising compound for the treatment of both androgen-dependent and independent prostate cancers.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10637-020-01050-w.

Acknowledgments Nanjing Sky Technology Co. Ltd. supported this study.

Author contributions Conceptualization: [Ahmed Elgehama]; Methodology: [Ahmed Elgehama]; formal analysis and investigation: [Ahmed Elgehama]; Writing–original draft preparation: [Ahmed Elgehama]; Writing - review and editing: [Guo Wenjie, Qiang Xu]; Funding acquisition: [Ahmed Elgehama, Qiang Xu]; Resources: [Li Junsun, Biao Yu, Qiang Xu, Ahmed Elgehama]; Supervision: [Qiang Xu].

Funding Nanjing Sky Technology Co., Ltd. Supported this study.

Data availability All data have been included in the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethics approval All experiments were performed following the ethical guidelines for scientific practices.

Consent to participate N/A

Consent for publication All authors approved the publication of this study.

Code availability N/A

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