## ORIGINAL ARTICLE

# Targeting castration-resistant prostate cancer with a novel RORγ antagonist elaiophylin

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**Abstract**   Prostate cancer (PCa) patients who progress to metastatic castration-resistant PCa (mCRPC) mostly have poor outcomes due to the lack of effective therapies. Our recent study established the orphan nuclear receptor RORγ as a novel therapeutic target for CRPC. Here, we reveal that elaiophylin (Elai), an

antibiotic from *Actinomycete streptomyces*, is a novel ROR$\gamma$ antagonist and showed potent antitumor activity against CRPC *in vitro* and *in vivo*. We demonstrated that Elai selectively binded to ROR$\gamma$ protein and potently blocked ROR$\gamma$ transcriptional regulation activities. Structure−activity relationship studies showed that Elai occupied the binding pocket with several key interactions. Furthermore, Elai markedly reduced the recruitment of ROR$\gamma$ to its genomic DNA response element (RORE), suppressed the expression of ROR$\gamma$ target genes *AR* and *AR* variants, and significantly inhibited PCa cell growth. Importantly, Elai strongly suppressed tumor growth in both cell line based and patient-derived PCa xenograft models. Taken together, these results suggest that Elai is novel therapeutic ROR$\gamma$ inhibitor that can be used as a drug candidate for the treatment of human CRPC.

## 1. Introduction

Metastatic castration-resistant prostate cancer (mCRPC) remains one of the leading causes of cancer-related death in men[1]. Next-generation anti-androgen therapeutics enzalutamide (ENZ) and abiraterone (ABI) are being used as one of the standard first-line therapies for the treatment of mCRPC and showing improvement of the disease outcomes in some patients. However, most tumors develop resistance and advance to incurable diseases[2,3]. One major mechanism of ABI or ENZ resistance is that tumor cells up-regulate the expression of androgen receptor (AR) variants (ARvs)[4−7]. Unlike AR full-length (AR-FL), most of the variants lack functional ligand binding domain (LBD) and thus are not responsive to anti-androgen therapies[7], which limits the significance of developing more potent anti-androgens. Therefore, new therapeutic strategies for metastatic castration-resistant PCa (mCRPC) are urgently needed.

The RAR-related orphan receptors (RORs) are members of the nuclear receptor superfamily and consist of ROR$\alpha$, $\beta$, and $\gamma$[8,9]. They control gene expression by directly binding to their corresponding genomic sites, and have different physiological functions. T cells have a specific ROR$\gamma$ isoform, namely ROR$\gamma$t, which differs in the N-terminal, less than 20 amino acids, due to T cell-specific promoter usage[7]. It is well established that ROR$\gamma$t plays a crucial role in inflammation-associated Th17 cell development and autoimmune diseases[10]. Intensive studies are focused on therapeutic development targeting it in autoimmune diseases[8,11−13]. However, the roles of ROR$\gamma$ in human diseases remain largely unclear. Recently, works from our group and others established that ROR$\gamma$ is potential therapeutic target for the treatment of cancers[14−16]. In CRPC tumors, ROR$\gamma$ is overexpressed and/or amplified, and functions as a key determinant of AR overexpression and aberrant signaling. The inhibition of ROR$\gamma$ strongly suppresses AR-FL and ARvs expression, and potently blocks CRPC cell growth *in vivo* and *in vitro*[14]. Thus, targeting ROR-$\gamma$ is a promising strategy for effective CRPC therapy and overcoming anti-androgen therapeutic resistance.

Natural products have been a major source of drugs for the treatment of various diseases for thousands of years[17,18]. More than 40% of antitumor drugs are developed from natural sources[19]. Compared with natural products from terrestrial life, discovery and utilization of marine natural products for drug development are uncommon. At present, only a handful of drugs from marine sources have been approved by U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA) and used in clinical treatment of diseases such as cancers[20]. Marine natural products have a high diversity of chemical structures, which are considered to be the most promising and sustainable medicine source. With the advancement of technology, the number of identified marine natural products has increased dramatically[21].

In this study, we demonstrated that elaiophylin (Elai), an antibiotic obtained from marine-derived *Actinomycete streptomyces* sp. SCSIO 41398[22], is a novel ROR$\gamma$ inhibitor and possesses a potent anti-tumor activity against CRPC *in vitro* and *in vivo* through suppressing the expression of AR-FL and ARvs. Our results suggest that Elai might be a drug candidate for the treatment of human CRPC.

## 2. Materials and methods

### 2.1. Cell culture and chemicals

22Rv1 and VCaP were from American Type Culture Collection (ATCC, Manassas, VA, USA). C4-2B was from UroCor Inc. (Oklahoma City, OK, USA). C4-2B and 22Rv1 cells were cultured in RPMI1640 medium, VCaP and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM). All culture media were supplemented with 10% fetal bovine serum and 1 × penicillin/streptomycin (Gibco, Grand Island, NY, USA). Cells were cultured at 37 °C in a humidified incubator containing 5% $CO_2$. Elaiophylin (Elai) was purchased from APExBIO (Houston, TX, USA) and ACMEC (Shanghai, China). Other chemicals were purchased from Sigma−Aldrich (St. Louis, MO, USA) unless specified otherwise.

### 2.2. Cell viability

Cells were seeded in 96-well plates at 500−1000 cells per well (optimum density for growth) in a total volume of 100 μL of media. Serially diluted compounds in 250 μL of media were added 50 μL to the cells per well 24 h later. After 4 days of incubation, Cell-Titer GLO reagents (Promega Corp., Madison, WI, USA) were added, and luminescence was measured on GLOMAX microplate luminometer (Promega Corp.) according to the manufacturer's instructions. The results were presented as percentages and vehicle-treated cells set at 100.

## 2.3. Colony formation

Colony formation was performed as described previously[23], 500 cells were seeded in each well of 6-well plates and cultured for 12−14 days with the medium changed as well as the compound added every 3 days. Cells were then fixed in 4% para-formaldehyde for 15 min. The plates were washed with PBS three times. Cell colonies were stained with crystal violet for 15 min. The numbers of colonies were counted after being washed three times with PBS.

## 2.4. Caspase-3/7 activity and cell growth

For apoptosis, caspase-3/7 activity was measured as in a previous report[16]. Briefly, caspase-3/7 activity was measured by using a luminescent caspase-Glo 3/7 assay kit (Promega Corp.) following the manufacturer's instructions. Cell protein concentration was quantified to normalize the results. For cell growth, cells were seeded in 6-well plates at $1.5 \times 10^5$ per well and treated as indicated. Total viable cell numbers were counted by a Coulter cell counter.

## 2.5. Surface plasmon resonance (SPR) analysis

SPR measurements were performed on a Biacore 8K instrument (GE Healthcare, Piscataway, NJ, USA). Briefly, purified RORγ and RORα (200 μg/mL, pH 8.0) were immobilized (∼10,000 RU) on a Series S Sensor Chip (GE Healthcare, Piscataway, NJ, USA) according to a standard amine coupling procedure. PBS (G0002, pH7.2−7.4; Servicebio, Wuhan, China) with 5% DMSO, was used as the running buffer for immobilization. After immobilization, the solution of Elai was prepared with running buffer through serial dilutions of stock solution. Nine concentrations of Elai were injected simultaneously at a flow rate of 65 μL/min for 60 s of association phase at 25 °C. The final graphs were obtained by subtracting blank sensorgrams. Experimental data were collected with the Biacore 8K manager software and were analyzed to fit to an appropriate binding model to obtain the equilibrium dissociation constant ($K_d$).

## 2.6. Transient transfection and luciferase assays

Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For luciferase reporter gene assays, 293T cells were seeded at $10^4$ cells per well in 96-well plates in phenol red-free DMEM medium (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) charcoal stripped fetal bovine serum and co-transfected with pBD-RORγ−LBD (25 ng per well) and pFR-luci (25 ng per well), with pcDNA-PGC-1α (10 ng per well) plasmids or PLX304-V5(N)-RORγ (25 ng per well) and pGL3-AR-RORE (25 ng per well) plasmids. Renilla luciferase plasmid (40 ng per well) was used as an internal control. Twenty-four hours after transfection, transfected cells were treated with Elai for another 24 h before cells were harvested for luciferase assays. Luciferase activity was performed following the Dual-Luciferase Reporter Assay System introduction (Promega Corp.).

## 2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as our previous work[14]. C4-2B cells were treated with vehicle or Elai (1 μmol/L) for 24 h before harvested for ChIP analysis. Anti-RORγ rabbit serum was described previously[14]. The primers are shown in Supporting Information Table S1.

## 2.8. qRT-PCR

Total RNA was extracted from cells in 6-well plates. cDNA was prepared according to the manufacturer's protocol. PCR was performed on a BIO-RAD CFX96™ (BIO-RAD, San Diego, CA, USA) in the presence of SYBR Green. After the fluorescence values were collected, a melting-curve analysis was performed. The experiments were performed at least three times, with data presented as mean ± standard deviation (SD). The sequences of primers for the qRT-PCR analysis are listed in Supporting Information Table S2.

## 2.9. Western blotting

Western blotting was performed as in our previous work[23,24]. Antibodies specifically against RORγ, AR, and the other related proteins were used. All the antibodies used in this study are described in the Supporting Information Table S3.

## 2.10. Molecular docking

The crystal structure of RORγ in complex with the antagonist XY101 (PDB code: 4J1L.pdb) was downloaded from Protein Data Bank (http://www.pdb.org). The antagonist XY101 was removed from the protein structure, the hydrogen atoms and disulfide bonds of the protein were added by AmerTools. To obtain a large binding pocket for the two compounds Elai and halichoblelide D (Hali), we first performed a 100-ns classical molecular dynamic (MD) simulation for the protein following the same protocol as in our previous studies[25,26] by using the AMBER12 package[27]. Then we chose the snapshot from the 40-ns MD simulation for the subsequent molecular docking modeling. The molecular docking was performed by Schrödinger program following our previous study[28]. The ligand and protein parameters for docking were prepared by Maestro (version 10.1, Schrödinger, LLC, New York, NY, 2015). The Glide docking program in Maestro 10.1 was used for docking studies. For Glide docking, the grid was defined using a 30 Å box centered on residue MET365 of RORγ, and the important water molecules around the ligand were kept. All parameters were kept as default. The designed molecules were docked using Glide XP mode, and the predicted binding modes of both Elai and Hali were ranked according to their glide scores.

## 2.11. Animal experiments

Four-week-old male NOD/SCID mice (body weight ∼18 g) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Briefly, $1 \times 10^7$ 22Rv1 cells were suspended in total of 100 μL PBS and matrigel (1:1), and implanted subcutaneously into the dorsal flank of the mice. When the tumor volume was reached about 50 mm³, the mice were grouped randomly. Then mice were divided into two groups ($n > 6$) randomly and treated intraperitoneally (i.p.) with 100 μL of either vehicle or Elai (2 mg/kg) for five times per week. Tumor volume and body weight were measured two times per week. The volume was calculated with Eq. (1):
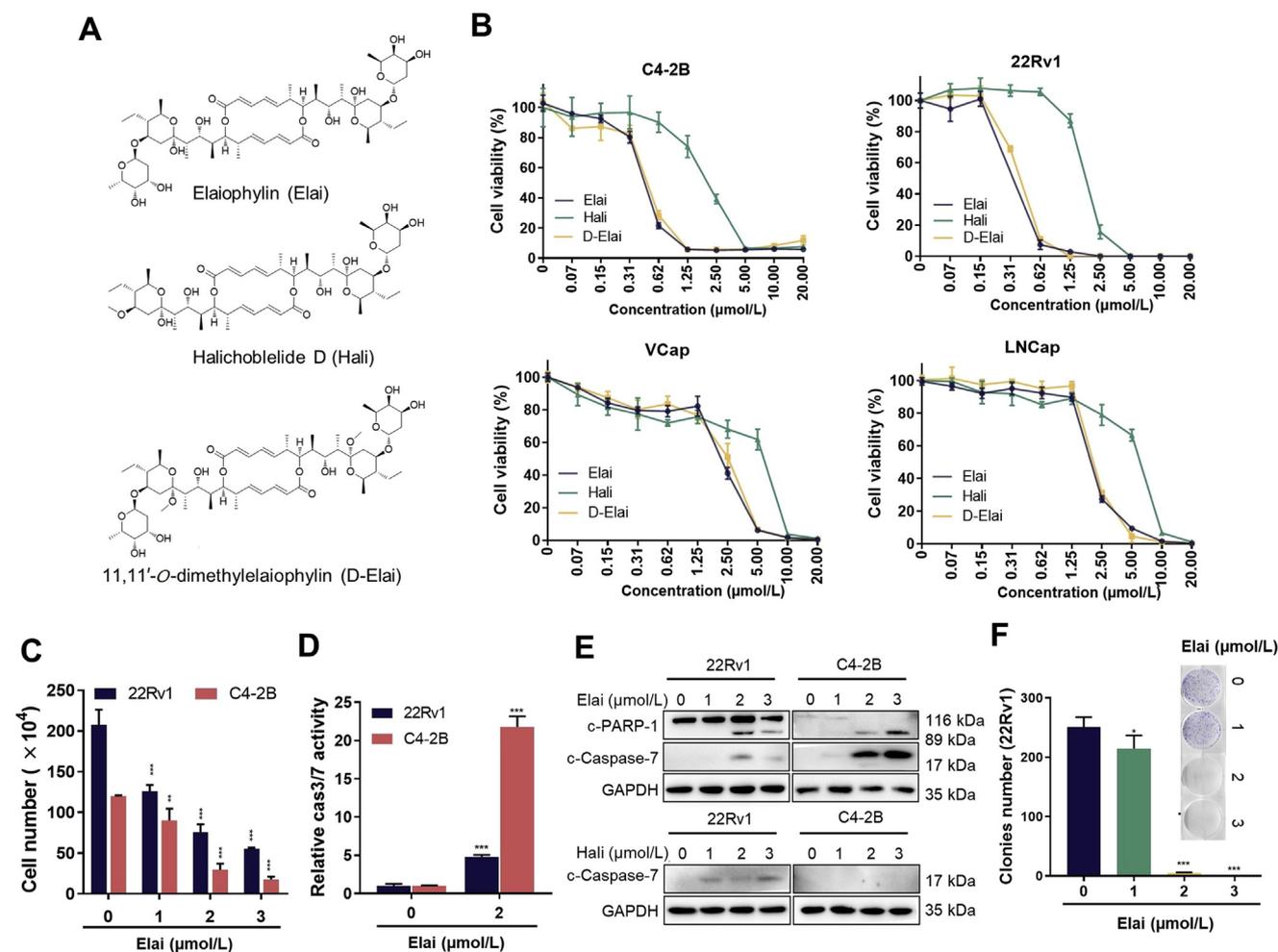
**Figure 1**    Elai inhibits castration-resistant prostate cancer cell survival and induces their apoptosis. (A) Chemical structures of elaiophylin (Elai), halichoblelide D (Hali) and 11,11'-$O$-dimethylelaiophylin (D-Elai). (B) Cell viability were measured by Cell-Titer GLO (Promega) of C4-2B, 22Rv1, VCaP and LNCaP cells treated with the indicated concentrations of Elai, Hali and D-Elai for 4 days. Experiments were performed three independent times and sextuplicate. (C) 22Rv1 cells and C4-2B cells were treated with vehicle or Elai as indicated. After 24 h, total viable cells were counted with a Coulter cell counter. (D) 22Rv1 and C4-2B cells were treated with vehicle or Elai for 24 h before collected for apoptosis analysis by measuring caspase-3/7 (Cas3/7) activities. (E) Immunoblotting analysis of the indicated proteins in 22Rv1 and C4-2B cells treated with vehicle or Elai or Hali for 24 h. (F) 22Rv1 cells were treated with vehicle (DMSO) or the indicated concentrations of Elai for 12 days, after which colony formation was assessed. All data shown above are mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* control.

$$V = \pi/6 \ (\text{Length} \times \text{Width}^2) \qquad (1)$$

The mice were sacrificed at the end of the studies. Tumors were harvested, weighted and analyzed by immunohistochemistry and immunoblotting assays as previous report[14]. The patient-derived xenograft (PDX) tumor model, PDX-TM00298, was purchased from the Jackson laboratory. The PDX tumors were propagated in the dorsal flank on both sides of the mice. The effect of Elai on PDX tumor growth was evaluated as above description. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (Guangzhou, China).

### 2.12.    Statistical analysis

All analyses were conducted with GraphPad Prism7 (GraphPad Software, USA). The data are presented as mean ± SD from three independent experiments. Statistics analysis was performed using two-tailed Student's $t$-tests to compare the means. $P < 0.05$ was considered to be significant.

### 3.    Results

#### 3.1.    Elai inhibits castration-resistant prostate cancer (CRPC) cell survival and induces their apoptosis

To search for drug candidates for the treatment of human CRPC, we screened a chemical library, which contains 500 marine natural compounds, for their activities in growth inhibition of a CRPC cell line C4-2B. Elaiophylin (Elai) and its derivative 11,11'-$O$-dimethylelaiophylin (D-Elai) exhibited a strong activity in inhibition of C4-2B cell survival, while halichoblelide D (Hali) showed less potent inhibition (Fig. 1A and B). Moreover, strong growth inhibition by Elai was observed in other prostate cancer cell lines
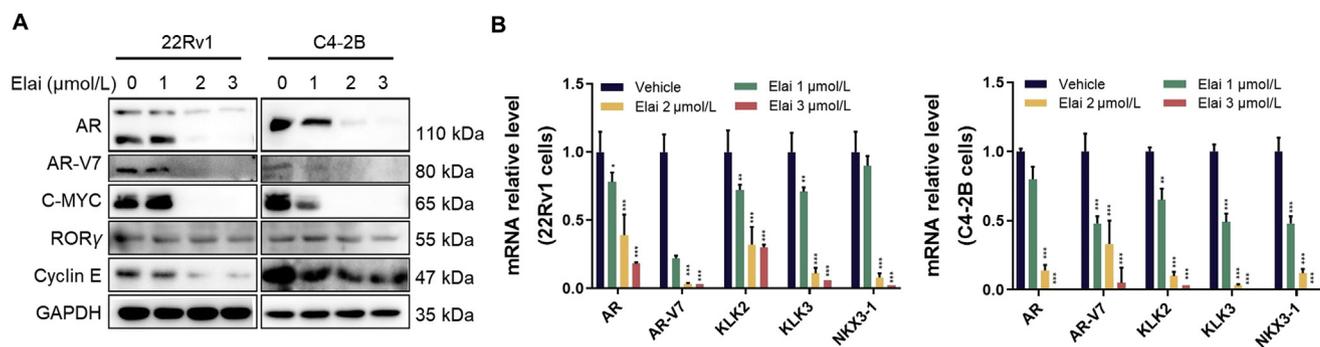
**Figure 2** Elai inhibits AR expression and signaling. (A) Immunoblotting analysis of the indicated proteins in 22Rv1 and C4-2B cells treated with vehicle or Elai for 24 h. (B) qRT-PCR analysis of the indicated genes in 22Rv1 and C4-2B cells treated with vehicle or Elai for 24 h. All data shown above are mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control.

22Rv1, VCaP and LNCaP cells at nanomolar concentration (Fig. 1B and C). We also found that Elai could strongly induce apoptosis of 22Rv1 and C4-2B cells, as demonstrated by measuring caspase-3/7 (Cas3/7) activities (Fig. 1D). Also, the expression of apoptosis-related proteins such as cleaved-PARP-1 and cleaved-caspase 7 were increased after treatment with Elai (Fig. 1E). Consistent with the effects of Elai on CRPC cell growth and apoptosis, Elai potently reduced CRPC cellular colony formation (Fig. 1F and Supporting Information Fig. S1). These data suggest that Elai inhibits CRPC cell survival and induce their apoptosis in vitro.

### 3.2. Elai inhibits the expression of AR-FL and AR-V7 and AR-mediated signaling

Since androgen receptor (AR) and its variants are highly over-expressed in the majority of CRPC tumors and play a pivotal role in the PCa development and progression, we thus examined whether Elai inhibited CRPC cell survival via blocking AR signaling. Indeed, we observed that Elai significantly inhibited the expression of AR and its variants at both mRNA and protein level in a dose-dependent manner (Fig. 2A and B). Moreover, Elai decreased the expression of proliferation and survival proteins
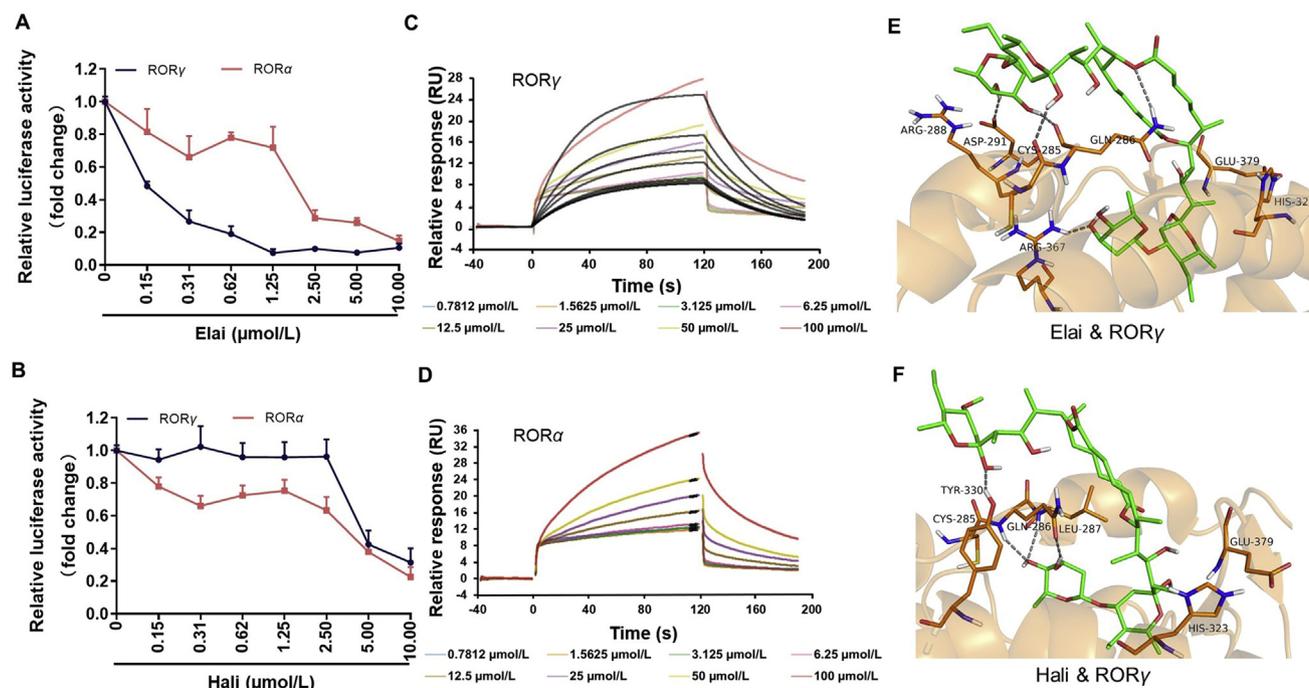


**Figure 3** Identification of Elai as a novel RORγ antagonist. RORγ and RORα LBD guide-gene reporter assays, with 293T cells showing the effects of Elai (A) and Hali (B) on RORγ and RORα activation. Fold change indicates the activities of RORγ and RORα under influence of Elai compared to control set as 1. The data are mean ± SD, $n = 3$. SPR analysis of the binding affinity of Elai to RORγ (C) and RORα (D) protein. Apparent equilibrium dissociation constants ($K_d$) were then calculated as the ratio of $k_d/k_a$. The $K_d$ (mol/L) value between Elai and RORγ is $5.05 \times 10^{-6}$, while the value for Elai and RORα is $2.62 \times 10^{-5}$. 3D presentation of the predicted binding mode of Elai (E) and Hali (F) with RORγ by molecular docking. The hydrogen bonds were labeled by dashed lines.

such as MYC and cyclin E. Further examination by qRT-PCR analysis showed that Elai strongly suppressed the expression of AR target genes *KLK3*, *KLK2* and *NKX3-1* (Fig. 2B). Therefore, these data suggest that Elai inhibits AR signaling pathway by suppressing the expression of *AR* gene.

### 3.3. Identification of Elai as a novel RORγ antagonist

Our previous study demonstrated that RORγ is a key determinant of *AR* gene expression[14]. This promoted us to examine whether Elai suppressed *AR* gene expression *via* inhibition of RORγ activity in CRPC cells. Using cell-based luciferase reporter assays, we found that Elai strongly suppressed RORγ activity while Hali was less potent (Fig. 3A and B), which is consistent with effects of compounds on CRPC cell survival. Moreover, Elai showed only moderate inhibition on RORα activities (Fig. 3A). Next, we performed surface plasmon resonance (SPR) assays to determine whether Elai would directly bind to the LBDs of RORs, and found that Elai had a high affinity to the RORγ protein ($K_d = 5.05 \times 10^{-6}$ mol/L) than RORα ($K_d = 5.05 \times 10^{-5}$ mol/L, Fig. 3C and D).

We then performed molecular docking to predict the binding mode and investigate the Elai−RORγ interaction details. Molecular docking as illustrated in Fig. 3E and F demonstrates that both Elai and Hali bound to the RORγ binding pocket as general antagonists[28] through several hydrogen bonds. For Elai, the tetrahydropyranol group inserted into the binding pocket only forms H-bond with ARG367, while the tetrahydropyranol group exposed in the solvent forms H-bonds with ASP291, CYS285 and GLN286. In addition, the exposed carboxylate oxygen atom in the big ring of Elai forms H-bond with GLN286. Whereas, for Hali, the inserted tetrahydropyranol group forms H-bonds with ASP291, CYS285 and GLN286, while the exposed group only forms H-bond with TYR330. Moreover, Elai inserted much deeper into the binding pocket than Hali, and the exposed groups of Elai bind tighter than those of Hali. In addition, the Glide XP score for the binding mode of Elai is higher than Hali (−9.44 *vs.*

−8.57 kcal/mol). These binding affinities were in line with the above inhibition of Elai and Hali D on RORγ activities. Taken together, our results suggest that Elai is a novel, selective and potent RORγ antagonist.

### 3.4. Elai inhibits AR gene expression and cell survival via suppressing RORγ activity

Our previous study demonstrated that RORγ could directly bind to an AR-RORE site in the first exon of *AR* gene to drive its expression[14]. Moreover, our RORγ ChIP-seq analysis also showed apparent RORγ occupancy peak at the AR-RORE site[16]. We thus performed ChIP-qPCR analysis and found that Elai strongly reduced RORγ binding to AR-RORE site (Fig. 4A). In addition, using a cellular AR-RORE driven gene report assay, we found that Elai significantly suppressed RORγ transcriptional activity in a dose dependent manner (Fig. 4B). To provide further evidence that Elai reduced cell viability *via* inhibiting RORγ activity, we used *RORC* siRNA approach to specifically silence RORγ and demonstrated that Elai-induced inhibition of cell survival was significantly attenuated in *RORC* siRNA treated cells compared to control cells (Fig. 4C). Moreover, RORγ silencing also blocked the inhibition of Elai on prostate cancer cell colony formation (Supporting Information Fig. S2). These data collectively suggest that Elai inhibits *AR* gene expression and cell survival *via* suppressing RORγ activity.

### 3.5. Elai inhibits CRPC cell autophagy via suppressing RORγ activity

It has been reported that Elai inhibited autophagic flux *via* increasing the amount of SQSTM1[29], meanwhile, our previous gene profiling study also revealed that inhibition of RORγ with selective antagonist SR2211 and XY011 strongly increased the expression of SQSTM1 in CRPC cells[14]. This promoted us to examine whether RORγ was involved in Elai induced inhibition of autophagic flux in CRPC cells. Firstly, we performed
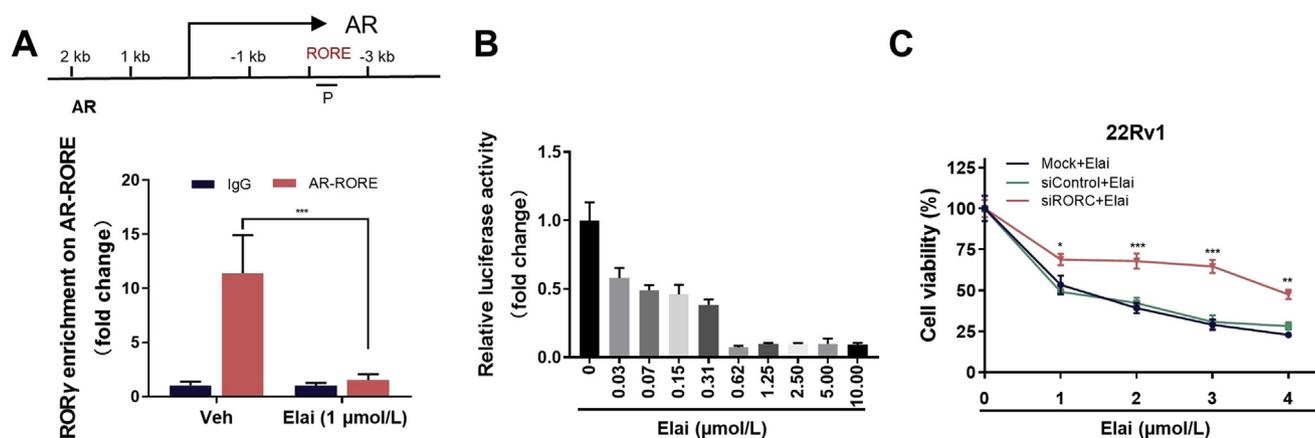


**Figure 4** Elai inhibits *AR* gene expression and cell survival *via* suppressing RORγ activity. (A) ChIP-qPCR analysis of relative enrichment of RORγ at AR-RORE site of *AR* gene in C4-2B cells treated with vehicle or Elai for 24 h. Fold change means the indicated enrichment on AR-RORE under influence of Elai compared to the IgG enrichment in cells treated with vehicle control set as 1. (B) AR-RORE driven gene report assay with 293T cells showing the effects of Elai on RORγ transcriptional activity. Fold change indicates the activities of RORγ under influence of Elai compared to control set as 1. (C) 22Rv1 cells were transfected with *RORC* or control siRNA for 48 h and treated with vehicle or Elai for another 24 h. Cells were harvested for determining cell growth by counting viable cells. All data shown above are mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* control.
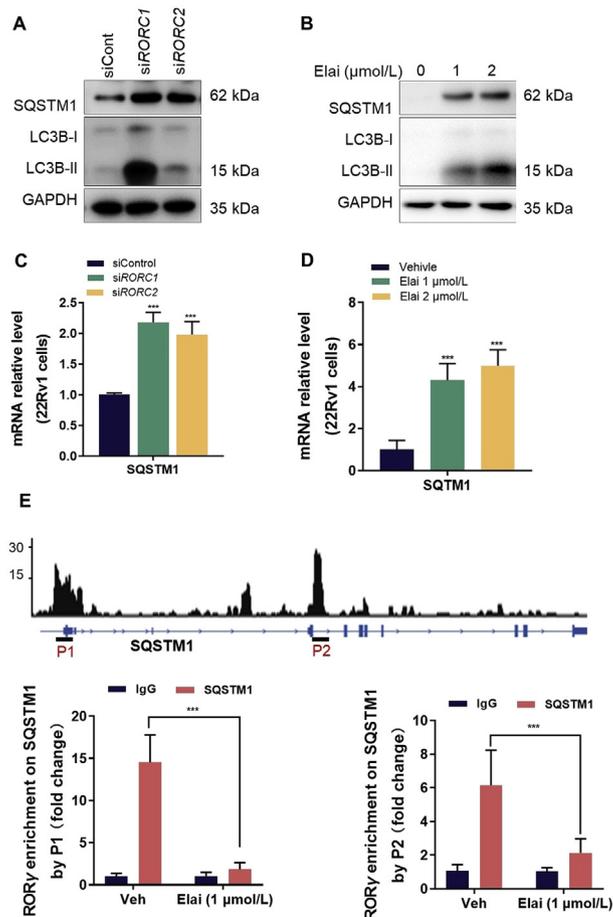
**Figure 5** Elai inhibits CRPC cell autophagy *via* suppressing RORγ activity. (A) 22Rv1 cells were transfected with control or *RORC* siRNA and cultured for 3 days before collected for immunoblotting with specific antibodies against indicated proteins. (B) Immunoblotting analysis of the indicated proteins in 22Rv1 cells treated with vehicle or Elai for 24 h. (C) 22Rv1 cells were transfected with control or *RORC* siRNA and cultured for 2 days before collected for qRT-PCR analysis of the indicated genes. (D) qRT-PCR analysis of the indicated genes in 22Rv1 cells treated with vehicle or Elai for 24 h. (E) Genome browser display of RORγ-binding events on promoter and body of *SQSTM1* gene, data from our previous ChIP-seq data (GSE126380) (upper) ChIP-qPCR analysis of relative enrichment of RORγ at *SQSTM1* gene promoter in C4-2B cells treated with vehicle or Elai for 24 h (bottom). Fold change means the indicated enrichment on *SQSTM1* gene under influence of Elai compared to the IgG enrichment in cells treated with vehicle control set as 1. All data shown above are mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* vehicle control.

immunoblotting for LC3B to validate the effects of RORγ knockdown and Elai on autophagy. Our results show that silencing RORγ with specific *RORC* siRNA significantly increased LC3B-II accumulation in CRPC cells. Interestingly, Elai treatment resulted in a similar increase of LC3B-II expression (Fig. 5A and B). Moreover, both Elai and RORγ knockdown significantly increased the expression of SQSTM1 at both mRNA and protein level (Fig. 5A−D). Since our previous ChIP-seq data showed that the promoter and enhancer regions of *SQSTM1* gene were occupied by RORγ protein[16] (Fig. 5E, upper panel), we performed ChIP-qPCR analysis to determine whether Elai treatment affects RORγ binding to the *SQSTM1* gene regulatory sites and found that Elai strongly reduced RORγ occupancy on the *SQSTM1* promoter (P1) and enhancer (P2) sites (Fig. 5E). Taken together, these data demonstrated that Elai might inhibit CRPC cell autophagy *via* increasing RORγ mediated *SQSTM1* gene expression.
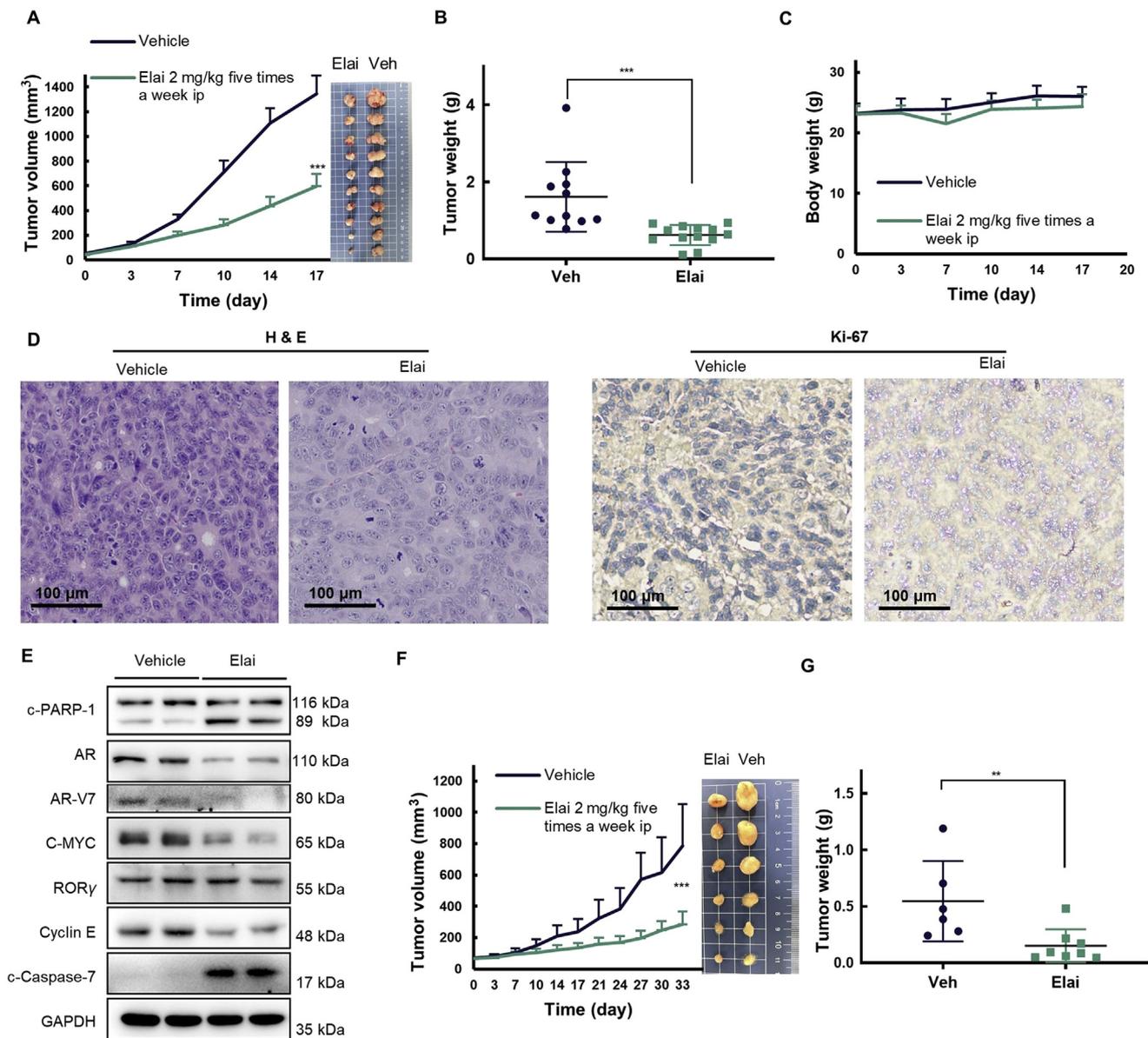
### 3.6. Elai inhibits CRPC tumor growth in vivo

We next assessed the effects of Elai on prostate cancer tumor growth. Elai (2 mg/kg, five times per week) or vehicle was intraperitoneally injected into mice bearing 22Rv1 xenograft tumors. Tumor volume and body weight were monitored twice a week. Results showed that Elai strongly suppressed tumor growth compared with vehicle group, and did not cause any significant change in mice body weight (Fig. 6A−C). Immunohistochemical Ki67 staining of the tumor sections showed that Elai significantly inhibited cancer cell proliferation (Fig. 6D). Meanwhile, immunoblotting analysis of xenograft tumors demonstrated that Elai significantly decreased the expression of AR and AR-V7, and increased the level of cleaved-caspase 7 and cleaved-PARP-1 which indicated induction of apoptosis (Fig. 6E). We also investigated the effect of Elai on autophagy in the xenograft tumors and found that Elai inhibited autophagy significantly with the

accumulation of LC3B-II (Supporting Information Figs. S3A and S3B), which indicated that the inhibition of autophagy might play a role in Elai blocking CRPC tumor growth. Moreover, we examined the effect of Elai on a patient derived prostate cancer xenograft model (PDX), results also showed that Elai significantly suppressed the tumor growth of this PDX model (Fig. 6F and G). These results demonstrated that Elai might be a drug candidate for prostate cancer therapy.

## 4. Discussion

Although significant progress has been made in the field of anti-androgen therapies for CRPC, *de novo* and acquired resistance to these therapies appear inevitable[30]. Development of novel therapeutic agents based on new targets may be able to overcome current CRPC resistance. Our recent study identified ROR$\gamma$ as a novel therapeutic target for CRPC[14]. Here, we demonstrated that



**Figure 6** Elai inhibits CRPC tumor growth *in vivo*. SCID mice bearing 22Rv1 xenografts received Elai (intraperitoneally i.p., 2 mg/kg) or vehicle for five times per week. Mean tumor volume±standard error of mean (SEM) (A), mean tumor weight±SEM (B), mean body weight±SEM (C) and representative tumor images (A) are shown. ***$P < 0.001$ *vs.* vehicle control. (D) Anti-Ki67 immunohistochemistry images and H&E staining of tumor section were shown. (E) Immunoblotting analysis of 22Rv1 xenograft tumors after 17 days of treatment with vehicle or Elai as in (A). SCID mice bearing prostate cancer patient derived xenografts (PDX) received Elai (intraperitoneally i.p., 2 mg/kg) or vehicle for five times per week. Mean tumor volume±SEM (F), mean tumor weight±SEM (G) and representative tumor images (F) are shown. **$P < 0.01$ *vs.* vehicle control.

Elai is a novel RORγ antagonist and that it showed potent anti-tumor activity against CRPC *in vitro* and *in vivo*.

Since AR plays crucial roles in the vast majority of PCa development and progression[31−34], current therapeutic development for CRPC still targets the androgen−AR axis *via* suppressing androgen production or blocking AR activation. However, high potent agents targeting the steps after *AR* gene being overexpressed cannot overcome AR variants induced therapeutic resistance. Our previous report has revealed that RORγ inhibition effectively blocked CRPC tumor growth *via* directly suppressing the expression of AR and its variants[23]. Here, we identified Elai as a novel RORγ antagonist. SPR analysis and gene report assays demonstrated that Elai selectively bound to RORγ protein and potently blocked RORγ activation. In CRPC cells, Elai markedly suppressed the expression of RORγ target genes *AR/AR* variants and significantly inhibited PCa cell growth *via* suppressing RORγ activity. Moreover, employing both cell line-derived based and patient-derived PCa xenograft models, we further demonstrated that Elai strongly suppressed PCa tumor growth *in vivo*.

Our gene expression profiling data showed that the impact of RORγ inhibition on tumor growth was unlikely to be limited to suppressing AR and its programs[14,16]. In addition to reducing AR levels, RORγ inhibition also increased the expression of autophagy adaptor SQSTM1[14] which was proved to inhibit autophagic flux and benefit CRPC therapy[35,36]. These promoted us to examine whether RORγ was involved in mediating CRPC cell autophagy. Indeed, we found that both Elai significantly blocked CRPC cell autophagy *via* increasing RORγ mediated *SQSTM1* gene expression, which indicated that the inhibition on autophagy also played a role in RORγ inhibitor blocking CRPC tumor growth. It was in line with previous reports, in which Elai increased expression of SQSTM1 and acted as an autophagy inhibitor to exert anti tumor activity in ovarian cancer cells[29]. These data suggest that Elai, as a novel RORγ inhibitor, might also exert anti-tumor activity through inhibiting autophagic flux. However, the exact role of autophagy in RORγ inhibition suppressed CRPC tumor growth remained to be further investigated.

## 5. Conclusions

We demonstrate here that Elai is a novel RORγ inhibitor. It strongly inhibited CRPC cell growth *in vitro* and *in vivo via* suppressing RORγ activity and downstream programs. Our data suggest that Elai could be used as a drug candidate for the treatment of human CRPC and might be able to overcome anti-androgen therapeutic resistance.

## Acknowledgements

## Author contributions

Junjian Wang, Fanghai Han, Yonghong Liu, Hong-Wu Chen, Peiqing Liu and Songtao Xiang designed experiments. Jianwei Zheng, Junfeng Wang, Qian Wang, Hongye Zou, Hong Wang, Zhenhua Zhang, Jianghe Chen, Qianqian Wang and Panxia Wang carried out experiments. Jing Lu, Xaiolei Zhang, Haibin Wang and Jinping Lei analyzed experimental results. Junjian Wang, Fanghai Han, Yonghong Liu, Hong-Wu Chen, Peiqing Liu, Jianwei Zheng and Yueshan Zhao wrote the manuscript. All authors have given approval to the final version of the manuscript.

## Conflicts of interest

The authors declare no conflicts of interest.

## Appendix A.    Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.07.001.

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