Enzalutamide (ENZ) is a second-generation androgen receptor (AR) antagonist used for the treatment of castration-resistant prostate cancer (CRPC) and reportedly prolongs survival time within a year of starting therapy. However, CRPC patients can develop ENZ resistance (ENZR), mainly driven by abnormal reactivation of AR signaling, involving increased expression of the full-length AR (ARfl) or dominantly active androgen receptor splice variant 7 (ARv7) and ARfl/ARv7 heterodimers. There is currently no efficient treatment for ENZR in CRPC. Herein, a small molecule LLU-206 was rationally designed based on the ENZ structure and exhibited potent inhibition of both ARfl and constitutively active ARv7 to inhibit PCa proliferation and suppress ENZR in CRPC. Mechanically, LLU-206 promoted ARfl/ARv7 protein degradation and decreased ARfl/ARv7 heterodimers through mouse double minute 2-mediated ubiquitination. Finally, LLU-206 exhibited favorable pharmacokinetic properties with poor permeability across the blood–brain barrier, leading to a lower prevalence of adverse effects, including seizure and neurotoxicity, than ENZ-based therapies. In a nutshell, our findings demonstrated that LLU-
1. Introduction

Prostate cancer (PCa) is the second leading cause of cancer-associated death in males worldwide. According to GLOBOCAN, an estimated 1.4 million new PCa cases and 375,000 deaths were found in 2020. Given that the prostate relies on androgens for its growth and development, androgen deprivation therapy is generally the primary choice for managing advanced PCa. However, almost all patients relapse and develop lethal castration-resistant prostate cancer (CRPC) within one year following androgen deprivation therapy as a result of the restart of androgen receptor (AR) signal pathways. Enzalutamide (ENZ) is widely utilized in clinical practice to treat CRPC as a second-generation AR antagonist by binding to AR’s ligand-binding domain (LBD).

Notwithstanding that ENZ can be efficient in the short term, an increasing body of evidence suggests that prolonged inhibition of AR signaling using ENZ triggers compensatory adaptive responses and drives CRPC progression to ENZ resistance (ENZR), which represents a significant challenge in the management of CRPC. Interestingly, multiple clinical trials suggested that 20%–40% of CRPC patients are inherently resistant to ENZ treatment. Accordingly, new insights into the underlying mechanisms of ENZ resistance may help provide optimal treatment in patients at risk of ENZR in clinical practice.

Frequently observed mechanisms underlying CRPC ENZ resistance are mainly driven by aberrant AR signaling, including AR amplification and overexpression (50% patients), generation of AR spliced variants (ARVs, 39% patients) or mutations in the LBD (15%–20% CRPC cases). As the most extensively characterized AR spliced variant, the ARV7 protein (or AR3) is the only quantifiable target with the greatest clinical and functional relevance to CRPC. In this regard, a study by Robinson et al. documented upregulated ARV7 expression in more than 90% of CRPC patient samples. Moreover, clinical studies have revealed that 50%–70% of ENZ-resistant patient samples exhibited at least a 20-fold increase of ARV7 expression (together with ARf), which was associated with poor prostate-specific antigen (PSA) progression-free survival.

Increasing evidence suggests that full-length AR (ARf) and ARV7 drive ENZR CRPC development by binding to chromatin in a codependent manner. Given that ARV7 lacks a nuclear localization signal, modulation of constitutive nuclear localization and transcriptional activity is dependent on the formation of heterodimers ARV7/ARf in an androgen-independent manner. Moreover, when ARf is co-expressed with ARV7, ARf can translocate to the nucleus without androgens. It is widely acknowledged that dimerization of ARf with ARf itself (homodimers) or ARV7 (heterodimers) is required for AR to regulate target gene expression. Interestingly, ARV7 can bind to ARf to repress transcription of growth-suppressive genes (e.g., SLC30A7, B4GALT1 and SNX14) that enable PCa cells to survive under ENZ treatment. Unfortunately, all clinically licensed drugs approved by FDA (e.g., ENZ, apalutamide (ARN-509) and darolutamide (ODM-201)) only modulate AR activity and therefore have no activity against ARf or ARf-mediated oncogenic signaling. Given this background, identifying novel AR antagonists that can disrupt the interaction between ARf and ARV7 by targeting ARf/ARV7 heterodimers can be effective in preventing CRPC progression and overcoming ENZR.

In the present study, through a series of screening and validation assays, we identified LLU-206, a novel ARf/ARV7 inhibitor with significant inhibitory activity on PCa cell proliferation and ENZ-resistant tumor growth. We further demonstrated that LLU-206 promoted ARf/ARV7 degradation and repressed the formation of heterodimers and transcriptional activities through the mouse double minute 2 (MDM2)-mediated ubiquitin–proteasomal system. Furthermore, we evaluated the drug-like properties of LLU-206, including the solubility, pharmacokinetics, bioavailability and toxicity. Overall, we provide experimental evidence that LLU-206 is a promising lead compound to overcome resistance to second-generation AR-targeted therapy.

2. Materials and methods

2.1. Cell lines, reagents and compounds

LNCaP, PC3 and 22RV1 cells (Chinese Academy of Sciences) were cultured using RPMI1640 containing 10% serum. Myc-CaP cells were obtained from Shanghai Jiao Tong University (Shanghai, China). HEK293T was provided by the Stem Cell Bank and cultured using DMEM containing 10% serum. Androgen deprivation was conducted by culturing cells in RPMI1640 (5% charcoal-stripped serum) for 48 h. ENZ (Cat# HY-70002), actinomycin D (ACTD) (Cat# HY-17559) and cycloheximide (CHX) (Cat# 66-81-9) were purchased from MCE. ARN-509 (Cat# 956104-40-8), ODM-201 (Cat# 1297538-32-9), MG132 (Cat# 1211877-36-9), dihydrotestosterone (DHT, Cat# 521-18-6), Nutlin3 (Cat# 890090-75-2) and R1881(Cat# 965-93-5) were obtained from Selleck. Recombinant Human MDM2/HDM2 Protein was obtained from R&D.

2.2. Western blot

Cell proteins were extracted using RIPA lysis buffer. Nucleus and cytoplasm proteins were extracted according to instructions of the corresponding extraction kit (Beyotime, Cat# P0028). The specific experimental procedures were performed as previously described, with primary antibodies including ARf (Abcam, Cat# ab74272), ARV7 (CST, Cat# 19672S), ARVs (Abcam, Cat# ab108341), PSA (CST, Cat# 5877S), Bax (Wanlei, Cat# W01637A), Bcl-2 (Proteintech, Cat# 60178-1-lg), and Bcl-1 (Proteintech, Cat# 60178-1-lg).
cleaved caspase 3 (Wanlei, Cat# WL03339a-020), MDM2 (CST, Cat# 86934S), Ubiquitin (Ub, Santa Cruz, Cat# sc-8017). Protein bands were detected using ECL (FDbio-Pico, Cat# FD8000).

2.3. RNA sequence and GSEA

22RV1 cells were administrated with LLU-206 or ENZ (10 μmol/L, 24 h, respectively), and the total RNA was collected by trizol for

Figure 1  Screening and identification of a novel small molecule inhibitor targeting AR. (A) Pyramid chart of the screening inhibitors of ARfI and ARv7 from the small molecule compounds library. (B) Three parental compounds were obtained by computer-aided drug design. (C) Effects of the top 8 compounds on cell viability in 22RV1 cells (n = 3). (D–F) LNCaP (ARfI positive cells), 22RV1 (both ARfI and ARv7 positive cells) and PC3 (transfected with ARfI and ARv7 expressing plasmids) cells were administrated with the top eight compounds (10 μmol/L, 48h). Moreover, the protein levels of ARfI, ARv7 and PSA were analyzed by Western blot. (G) The chemical structure of LLU-206. (H) Binding model of LLU-206 in AR-LBD binding pocket. (I) The docking prediction of LLU-206 (purple) with AR-LBD (blue). (J) The interaction between AR-LBD protein and LLU-206 was measured by biacore. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. n.s., not significant.
Figure 2  LLU-206 inhibits the proliferation and promotes the apoptosis of PCa and CRPC cells. (A) IC_{50} values of LLU-206 and ENZ on cell viability in different PCa cells treated for 72 h (LNCaP, 22RV1, LNCaP-ENZR and LNCaP-ARflOE). (B) The viability of LNCaP cells was evaluated after exposure to LLU-206 treatment at different concentrations with or without stimulation of DHT (10 nmol/L, 72h), while ENZ, ARN-509 and ODM-201 were used as control. (C) 22RV1 cells were treated with the indicated concentrations of LLU-206 and 10 μmol/L ENZ for seven days, and the colony formation assay was counted and quantified. (D) After being cultured for 9 days, the spheroids were treated with 10 μmol/L LLU-206, ODM-201, ARN-509 and ENZ for another five days. The spheroids were recorded on Days 11, 13 and 14. (E) The CD44 mRNA expression of the spheroids was detected by qPCR. (F) The apoptosis of 22RV1 cells was determined by flow cytometry after treatment with indicated drugs.
transcriptional sequencing (Novogene, China). Based on the data sets of RNA sequence, the KEGG-cell-cycle pathway was implemented in the gene set enrichment analysis (GSEA). HALLMARK Androgen Response gene signatures were obtained from GSEA official website (http://software.broadinstitute.org/gsea/index.jsp). Androgen-induced gene signatures were obtained from the study by Zhang et al.\(^2\), and the AR/ARv7-activated gene sets were derived from Cato et al.\(^3\).

2.4. Luciferase analysis

AR-deficient PC3 cells were transfected with ARF or expressing plasmids alone or PSA-promoter Firefly luciferase and Renilla luciferase reporter plasmids. After transfection for 24 h, different concentrations of LLU-206 and ENZ were added for another 24 h. Finally, the Duo-Lite Luciferase Assay System (Vazyme, Cat# DD1205) was used for the assay, and a multifunctional microplate reader was used for final measurements.

2.5. Immunoprecipitation assay (IP)

The cell lysis buffer for Western and IP (Beyotime, Cat# P0013) assay kit was used to extract the protein from the LLU-206 (10 μmol/L treatment group for 24 h. Whole-cell lysis was used for immunoprecipitation. Anti-AR or anti-ARv7 and homotypic control normal rabbit IgG (CST, Cat# 2729S) were added to the lysed proteins after being treated by magnetic beads protein A/G (Bimake, Cat# B23201) to remove proteins with nonspecific binding and incubated overnight, for at least 12 h on a horizontal shaker. Then, new magnetic beads were added into the antibody-coupled protein for 4 h in a rotator at 4 °C. The immunocomplexes were eluted by 2 \(\times\) loading buffer and detected by Western blot assay using the following antibodies (ARf, ARv7, Ub and MDM2).

2.6. Histology and immunohistochemistry

Histology and immunohistochemistry assays were performed as previously described\(^2\). Paraffin-embedded tissues were stained with Ki-67 antibody (CST, Cat# 12202S). Images were taken using a microscope. Ki-67 expression and necrotic tumor area were quantified by Image J and Image-Pro Plus software, respectively.

2.7. Bimolecular fluorescence complementation (BiFC) assay

PC3 cells were cotransfected with different BiFC fusion constructs. After 24 h transfection, the cells were administrated with R1881 (10 nmol/L) and LLU-206 (10 μmol/L) for another 24 h. Subsequently, the samples were trypsinized, and a green fluorescent protein fluorescence was observed and recorded by fluorescence microscopy (Leica). Flow cytometry was used to quantify the number of BiFC signals, and Graphpad compared the diversity.

2.8. Molecular docking and protein–ligand–protein interaction

The MDM2 (pdb: 3iwy) and AR-LBD (pdb: 4k7a) PDB files were downloaded from the Protein Data Bank (http://www.rcsb.org/). All heterogeneous atoms were removed. Then, 3iwy chain C and 4k7a chain A were selected for subsequent molecular docking. The MDM2 protein docking grid was maximized for LLU-206. Dynamic simulation PDB file (3iwy chain C) was converted to the PDBQT format as macromolecules before molecular docking. The grid (ligand docking search space) was located as described above. Then, Auto-dock Vina 1.1.2 was used for the subsequent molecular docking\(^3\).

GROMACS 5.1 was used to simulate protein–ligand–protein interaction between MDM2/LLU-206 complex and AR-LBD. Molecular dynamic simulation was performed for 500 ns or until the RMSF converged to a plateau. Then, protein–ligand–protein interaction was visualized using Pymol version 1.7.4.5. The amino acid residues of MDM2 and AR-LBD interacting with LLU-206, which are close to each other (≤1 Å), were selected for potential interactive fragment for subsequent verification.

2.9. Tumor xenografts

NOD-SCID and BALB/c-nude mice (6–8 weeks) were bought from GemPharmatech Co., Ltd. (Nanjing, China) and Charles River Laboratories (Shanghai, China), respectively. All animal procedures were conducted with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Research Ethics Committee of the Center for New Drug Evaluation and Research (No. B20180901-1).

2.10. Statistical analysis

The data were analyzed by GraphPad Prism 8. \(P\) values were compared using one-way ANOVA corrected with Tukey’s multiple comparisons test. A \(P\) value less than 0.05 was statistically significant. Data were expressed as mean ± standard error of mean (SEM).

3. Results

3.1. Screening and identification of a novel small molecule inhibitor targeting AR

To identify prominent AR inhibitors, 197 compounds with the novel scaffold of indoline thiohydantoin, tetrahydroisoquinoline thiohydantoin, and tetrahydroquinoline thiohydantoin were rationally designed based on the lead compound ENZ using the modification strategy of skeleton transition-merging ring\(^1\) (Fig. 1A and B). First of all, we assessed the viability of LNCaP, 22RV1 and LNCaP-ENZR cells with the treatment of 197 compounds (Supporting Information Fig. S1A). Eight compounds with LLU-206 and ENZ (10 μmol/L, 48 h). (G) GSEA analysis of the RNA sequence for KEGG CELL CYCLE in 22RV1 cells treated with LLU-206 (10 μmol/L, 24 h) and control (left). After 22RV1 cells were treated with LLU206 or ENZ (10 μmol/L, 48 h), the cell cycles were detected by flow cytometry (right). (H) The apoptosis markers cleaved caspase3, Bcl-2, Bax; proliferation makers AKT and p-AKT, ARF and ARv7 were analyzed by Western blot in 22RV1 cells treated with LLU-206 or ENZ for 48 h. Scale bar = 100 μm. Results are expressed as mean ± SEM, \(n = 3\). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\). n.s., not significant.
Figure 3  LLU-206 suppresses the growth of 22RV1 and LNCaP-ENZR xenografts in vivo. (A) LNCaP ENZR cells were generated by culturing the LNCaP cells in continuous exposure of ENZ for three months in vitro. (B) Mice bearing LNCaP-ENZR xenografts were administered with LLU-206 (10, 25 mg/kg) via tail vein and tumor volumes were measured every other day (n = 6). (C) Tumor weight of each group. (D) PSA levels of plasma were measured by ELISA kit. (E) Representative IHC staining of Ki-67. (F) Representative H&E staining of tumors. (G) The ARfl, ARv7, PSA protein levels in the tumors were detected by Western blot. (H) Representative bioluminescence images of 22RV1 xenografts in the prostate. (I) Tumor growth changes in mice bearing 22RV1 orthotropic tumors were analyzed by average fluorescence values (n = 6). (J) The relative tumor weight was measured at the end of the experiment in each group. (K) Representative H&E staining of 22RV1 tumor tissues were
(LLU-203, LLU-206, LLU-207, LLU-208, LS-13, LS-17, LDK-403 and LDK-404) showed relatively high anti-PCA activity. Importantly, significant inhibition of proliferation activities by LLU-206 in three ARf and/or Arv7-dependent PCA cells was observed, while no significant inhibition was observed with the second-generation U.S. Food and Drug Administration (FDA)-approved antagonists ENZ, ARN-509 and ODM-201 (Fig. S1A, Fig. 1C). The effect of the eight candidate compounds on ARf, Arv7 and Ar-activated PSA protein expression was further explored. Compared with ENZ and other antagonists, LLU-206 displayed excellent antagonistic activity against AR in Lncap, PC3-ARf-Arv7 (cotransfected with Arf and Arv7 plasmids) and 22RV1 cells, indicating that LLU-206 may be a potent AR inhibitor (Fig. 1D–F). The synthesis protocol and the main spectrum data of compound LLU-206 are displayed in Fig. S1B and S1C.

We further performed molecular docking studies and binding assays to improve our understanding of the interactions between LLU-206 and AR, including binding modes and binding site preference. Molecular docking prediction results showed that LLU-206 was tightly bound to AR-LBD and blocked the AR’s ligand-binding pocket (Fig. 1G and H). Additionally, based on the results from molecular docking calculation, we found that LLU-206 interacted with AR-LBD residues, such as GLN-42, PRO-13, VAL-15, PRO-132, ARG-83, ASN-87, TRP-82 and THR-86 (Fig. 1I). In contrast, only the nitrile nitrogen atom of ENZ could form two hydrogen bonds with Arg752 and Met749 (Fig. S1D).

To validate that LLU-206 could bind to AR, we constructed and purified the residues of AR-LBD that might be involved in the AR-LLU-206 interaction and performed SPR. Consistent with the molecular docking predictions, the direct binding assays showed a high binding affinity between LLU-206 and AR (KD = 558 nmol/L) (Fig. 1J). To further compare the affinity of LLU-206 and ENZ with AR-LBD, we detected the affinity between ENZ and AR-LBD. The binding assays showed that ENZ and AR-LBD had a lower affinity (KD = 44.4 μmol/L) than LLU-206 with AR-LBD, which suggested that LLU-206 had a higher affinity with AR than ENZ (Fig. S1E). Ultimately, we conducted acute toxicity testing to investigate whether LLU-206 exhibited any toxicity in vivo. During the experimental process, there were no physical signs of toxicity or poor condition in any experimental mice at the different dosage (up to 1200 mg/kg) (Supporting Information Table S1). Overall, the above findings suggest that LLU-206 is a lead candidate for preclinical development.

3.2. LLU-206 inhibits the proliferation and promotes the apoptosis of PCA cells

We further explored the effect of LLU-206 on the proliferation of ENZ-sensitive and ENZ-resistant CRPC cells in vitro. As shown in Fig. 2A and Supporting Information Fig. S2A, LLU-206 exhibited a higher degree of selective inhibition towards LNCaP cells (AR-rich) versus PC3 cells (AR-deficient) compared to ENZ. LLU-206 also effectively suppressed the growth of ARf and/or Arv7-driven ENZr cells (22rv1, Myc-CaP, LNCaP-ENZr and LNCaP-ARf106). Moreover, we further constructed an ENZ-sensitive CRPC cell model by artificially removing androgens from culture media that was then added back. The cell viability results showed that LLU-206 was more effective in inhibiting cell proliferation than AR antagonists ENZ, ARN-509 and ODM-201 in an androgen-independent manner (Fig. 2B, Fig. S2B and S2C). Besides, the colony formation experiment demonstrated that LLU-206 inhibited tumor growth in a dose-dependent manner (Fig. 2C and Fig. S2D). Consistently, the decrease of spheroids and CD44 expression after LLU-206 treatment also substantiated the depressive effect of LLU-206 on the stemness of PCA cells (Fig. 2D–E, and Fig. S2E). Further flow cytometry demonstrated an increase in apoptotic indexes (Fig. 2F and Fig. S2F). Interestingly, GSEA analysis revealed significant enrichment of pathways associated with cell cycle control after LLU-206 treatment, while cell cycle arrest at the G1 phase was documented by flow cytometry analysis (Fig. 2G and Fig. S2G). To further ascertain the effects of LLU-206, we also quantified the levels of apoptosis and AR-mediated proliferation-related proteins. Under different concentrations of LLU-206, a decrease in proliferation-related proteins in both AR signaling (ARf and Arv7) and AKT signaling [pAKT (308), pAKT (473) and AKT], and anti-apoptosis protein Bcl-2 (Fig. 2H) was observed. In contrast, the expression of apoptosis-associated proteins (Bax and cleaved caspase 3) increased. Overall, LLU-206 significantly inhibited malignant progression in various types of PCA cells, including ENZ-sensitive or ENZr CRPC cells. Importantly, we found that LLU-206 exerted more potent inhibitory effects than ENZ and other AR antagonists in vitro.

3.3. LLU-206 suppresses the growth of 22RV1 and LNCaP-ENZr xenografts in vivo

To assess the therapeutic potential of LLU-206 in models of ENZr xenografts, we first established an acquired ENZr cell line with increased expression of ARf and Arv7 protein using LNCaP cells exposed to increasing concentrations of ENZ (Fig. 3A). We measured the ARf/Arv7 expression levels of LNCaP cells exposed to ENZ for 3, 9, 10 and 12 months. As shown in Supporting Information Fig. S3A, the expression of ARf increased and the expression of Arv7 grew out of nothing and raised with the sustained exposure to ENZ. We further demonstrated that LNCaP-ENZr cells were completely resistant to ENZ in vivo (Fig. S3B). Then we tested the efficacy of LLU-206 in LNCaP-ENZr cells. As shown in Fig. 3B–D, LLU-206 exhibited significant antitumor activity in LNCaP-ENZr xenografts, as evidenced by the repressed tumor volume, tumor weight and serum PSA levels in a dose-dependent manner. Immunohistochemical (IHC) staining showed that Ki-67+ tumor cells were significantly decreased, while LLU-206-induced tumor necrosis was documented by hematoxylin-eosin (H&E) staining (Fig. 3E–F, and Fig. S3C). Furthermore, the protein expression of ARf, Arv7 and PSA. LLU-206 was significantly suppressed in tumors, indicating AR inhibition by LLU-206 in vivo (Fig. 3G). Taken together, these results indicated that LLU-206 exerted significant antitumor effects against xenografts with acquired resistance to ENZ.

To further assess the clinical potential of LLU-206, we compared its antitumor activity with ENZ in ARf/Arv7-driven
Figure 4  LLU-206 disrupts the ARfl/ARv7 heterodimers and the nuclear translocation. (A) The structure of ARfl and ARv7 comprises four functional domains: NTD (blue), DBD (red), hinge or CE3 (yellow) and the LBD (green). (B) A schematic diagram of the BiFC assay. Green fluorescence acquired with the dimerization of ARfl with ARfl or ARv7. (C) GFP fluorescence was determined by flow cytometry in PC3 cells cotransfected ARfl-C with ARfl-N or ARv7-N. (D) Co-IP assays with ARfl or ARv7 were performed using 22RV1 cells. The expression of ARfl and ARv7 was measured by Western blot. (E) The location of ARfl in LNCaP, treated with 5 μmol/L LLU-206 or ENZ for 24 h. (F) The location of ARfl and ARv7 in 22RV1 cells treated with LLU-206 or ENZ (5 μmol/L, 24 h, respectively). (G) The expression of ARfl protein levels in cytoplasm and nucleus of LNCaP cells treated with 10 μmol/L LLU-206 and ENZ for 24 h. (H) The expression of ARfl and ARv7 in cytoplasm and nucleus of 22RV1 cells treated with 10 μmol/L LLU-206 or ENZ for 24 h. Results are expressed as mean ± SEM, n = 3.
22RV1 orthotopic xenografts, which were inherently resistant to antiandrogen therapy (Fig. S3D). As shown in Fig. 3H–J, administration of 25 mg/kg/day LLU-206 significantly suppressed the growth of 22RV1 orthotopic xenografts (reduced the tumor weight by 49.4%), whereas the 22RV1 xenografts exhibited resistance to ENZ. In addition, LLU-206-treated tumors displayed a 30.80% decrease in the proliferative index and a 5-fold increase in apoptosis rate, as evidenced by Ki-67 staining and tumor tissue necrosis (Fig. 3K and L). Western blot analysis showed that ARfl, ARv7 and PSA protein levels were inhibited (Fig. 3M and N). Overall, the efficacy of LLU-206 in anti-PCa treatment was demonstrated in 22RV1 xenograft models with acquired resistance to ENZ.

In addition, no overt drug-related toxicity was observed in these two models at therapeutic doses of LLU-206 (Fig. S3E and S3F). H&E staining showed no significant injury was observed in vital organs such as the heart, liver, spleen, lung and kidney (Fig. S3G). Given the neurotoxicity of AR antagonists, the clinical safety profile was assessed by monitoring the incidence of seizures. Characterization of blood–brain barrier penetration was assessed by high-performance liquid chromatography assays. LLU-206 was not found in the mice brain while high concentrations of ENZ were present (Fig. S3H). The above findings suggest that LLU-206 not only inhibited tumor growth and malignancy in ENZ-resistant PCa models but also displayed low organ toxicity.

3.4. LLU-206 blocks ARfl/ARv7 heterodimer formation and nuclear transfer

It is widely acknowledged that dimerization of ARfl with ARfl itself (homodimers) or ARv7 (heterodimers) is required for AR to regulate target gene expression. Interestingly, ARfl/ARfl homodimers and ARfl/ARv7 heterodimers act as transcriptional activators of genes related to aggressive tumor growth, while ARfl/ARv7 heterodimers can also act as transcriptional repressors of growth-suppressive genes to sustain the progression of CRPC. Given the critical role of dimers in modulating AR function, the BiFC assay was used to detect whether LLU-206 attenuated dimers. For BiFC, if the two proteins were dimerized, the N- or C-terminal fragment interaction would allow generation of the Venus fluorescent protein to emit a fluorescent signal (Fig. 4A and B). To mimic AR expression (ARfl/ARfl homodimers and ARfl/ARv7 heterodimers) in clinical CRPC patients, PC3 cells were transfected with ARfl-VC plasmids combined with ARfl-VN or ARv7-VN plasmids. Fluorescent signals were then detected by flow cytometry and inverted fluorescence microscopy. The results substantiated that LLU-206 attenuated the formation of ARfl/ARfl homodimers and ARfl/ARv7 heterodimers (Fig. 4C, Supporting Information Fig. S4A and S4B). To further illustrate the effect of LLU-206 on the endogenous ARfl/ARv7 dimerization, we performed co-immunoprecipitation (co-IP) using anti-ARfl or anti-ARv7. We found that LLU-206 prevented the interaction between ARfl and ARv7 (Fig. 4D and Fig. 4C). Taken together, these data suggest that LLU-206 blocked ARfl/ARv7 heterodimer dimerization formation. Western blot and immunofluorescence assay further showed that both nuclear localization and the expression of ARfl and ARv7 were decreased following treatment with LLU-206 (Fig. 4E–H, and Fig. 5D). Collectively, these results indicate that LLU-206 disrupts the formation of ARfl/ARv7 heterodimers and ARfl/ARfl homodimers and further inhibits ARfl/ARv7 expression and nuclear transfer.

3.5. LLU-206 suppresses ARfl and ARv7 transcriptional activity

To examine whether LLU-206 affected ARfl or ARv7-dependent endogenous gene expression, we performed GSEA based on the transcriptional profiles of 22RV1 cells treated with LLU-206. GSEA revealed that the top-ranked gene sets enriched in 22RV1 control cells, including androgen response genes (P = 0.000, NES = 1.556, FDR = 0.115), androgen-induced genes (P = 0.000, NES = 1.518, FDR = 0.101), and ARfl/ARv7 activated genes (P = 0.000, NES = 1.518, FDR = 0.094) were suppressed by LLU-206 treatment, indicating that LLU-206 suppressed the AR signaling pathway that contributed to ENZ resistance in PCa cells (Fig. 5A). Analysis of RNA-seq data showed a series of AR activated and repressive genes were suppressed and upregulated after the treatment of LLU-206 (Fig. 5B and Supporting Information Fig. S5A).

Furthermore, the levels of mRNA transcripts for several well-characterized ARfl and ARv7-regulated genes were further evaluated in ENZ sensitive or resistant cells (Fig. 5C and D). Several ARfl and ARv7 activated genes that promote PCa proliferation, such as PSA and TMPRSS2, FBKBP5, KLK2 and UBE2C, were suppressed (Fig. 5C–E). Conversely, ARv7-suppressive genes that inhibit PCa proliferation (e.g., SLC30A7, B4GALT1 and SNX14) were significantly upregulated by LLU-206 (Fig. 5C and F). As shown in Fig. 5D, 5F and 5S, LNCaP and 22RV1 cells were treated with 10 μmol/L LLU-206 for 24 h. We found that LLU-206 did not affect the mRNA expression of ARfl/AR7. Moreover, when PCa cells were treated with RNA synthesis inhibitor ACTD or both ACTD and LLU-206, no difference in ARfl/AR7 mRNA stability was found between the two groups (Fig. 5D and S5E). These results collectively indicate that LLU-206 did not influence ARfl and ARv7 mRNA levels and stability. To further characterize whether LLU-206 repressed the transcriptional activities of ARfl and ARv7, we utilized luciferase reporter assays (ARfl-PSA-Luc system) to assess LLU-206 activity. As shown in Fig. 5G, LLU-206 dose-dependently antagonized the DHT-mediated transcriptional activities of ARfl, whereas ENZ was significantly less effective at low concentrations (0.3 μmol/L). These results collectively indicate that LLU-206 regulated ARfl/ARv7 transcriptional activity and hence contribute to inhibition of AR dimers-mediated gene expression.

3.6. LLU-206 induces degradation through MDM2-mediated ubiquitin–proteasomal system

Our results show that LLU-206 effectively reduced ARfl, ARv7, and PSA protein levels in a dose-dependent and time-dependent manner (Fig. 6A, Supporting Information Fig. S6A and S6B). To further investigate the mechanisms of LLU-206 in inhibiting ARfl/ARv7 expression, we explored the effect of LLU-206 on protein stability with a protein synthesis inhibitor CHX. Notably, ARfl and ARv7 protein degradation was significantly accelerated under LLU-206 treatment (Fig. 6B and C, Fig. 5C). In recent years, the ubiquitin/proteasome system has been found to play a significant role in degrading nuclear hormone receptors and regulating their transcriptional function. To validate whether LLU-206-mediated degradation was associated with the ubiquitin-proteasome pathway, we treated cells with the proteasome inhibitor MG132. The results show that LLU-206-induced ARfl/ARv7 depletion was
Figure 5  LLU-206 suppresses ARfl and ARv7 transcriptional function/activity. (A) GSEA analysis of the RNA sequence for AR response, AR-induced and AR/ARv7 activated gene sets in 22RV1 cells treated with LLU-206 (10 μmol/L, 24 h) and control. ES, enrichment score. (B) Classical AR activated and repressed genes were shown in the RNA sequence data. (C) The mRNA levels of AR-activated or repressed genes were detected in LNCaP or LNCaP-ENZR cells treated with 10 μmol/L LLU-206 for 24 h (n = 6). (D) The mRNA levels of AR and AR-activated gene were measured in LNCaP cells treated with 10 μmol/L LLU-206 for 24 h (n = 6). (E, F) The mRNA levels of ARfl and ARv7 activated or repressed genes were detected in 22RV1 cells treated with LLU-206 for 24 h. (G) A luciferase assay was conducted in PC3 cells treated with LLU-206 with or without 10 nm R1881 for 24 h after transfection with ARfl and PSA-promoter luciferase plasmid. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. n.s., not significant.
Figure 6  LLU-206 induces degradation through the MDM2-mediated ubiquitin–proteasomal system. (A) The protein levels of ARfl or ARv7 were detected in 22RV1 cells treated with LLU-206 (5 μmol/L, 10 μmol/L) and ENZ (10 μmol/L) for 48 h. (B) The effect of LLU-206 on ARfl and ARv7 stability was measured by Western blot. The protein levels were detected in 22RV1 cells, which were incubated with CHX (50 μg/mL) with or without LLU-206 for a different time. (C) Statistical analysis of the relative protein expression of ARfl and ARv7 for three independent experiments (n = 3). (D) Western blot assay for the protein levels of ARfl and ARv7 in 22RV1 cells, which were treated with LLU-206 (10 μmol/L, 24 h), and MG132 (10 μmol/L, 6 h). (E) IP was conducted using anti-ARfl and immunoblotting was performed with ARfl, ARv7
significant. MDM2, a well-known E3 ligase for AR ubiquitination, was identified as a potential target for LLU-206. We performed computer modeling docking studies to identify potential inhibitors with high binding affinity towards MDM2. The docking results showed that LLU-206 has a high binding affinity for MDM2, with a binding affinity score of -5.6 mol/L. The docking model of LLU-206 (purple), AR-LBD (blue) and MDM2 (green) is shown in Fig. S6H. The binding sites of LLU-206, AR-LBD and MDM2 are indicated in the heatmap (Fig. 6G). The docking results suggested that LLU-206 interacts with MDM2 with the highest binding affinity. In our study, the molecular docking prediction showed that LLU-206 may function as a molecular glue, which can disrupt the interaction between ARfl and MDM2, leading to protein degradation.

4. Discussion

Current standard-of-care therapy for CRPC includes second-generation non-steroidal antiandrogens ENZ, ARN-509 and ODM-201 that suppress androgen receptor signaling. These drugs can prolong patient survival, they are not curative and inherent or acquired therapeutic resistance remains a major clinical challenge. PCa is characterized by aberrant reactivation of AR signaling due to increased expression of AR and ARv7, a constitutively active LBD-deficient variant associated with poor prognosis and therapeutic drug resistance. Accordingly, identifying agents that can simultaneously target ARfl and ARv7 may provide a novel approach to overcome ENZR. In the present study, we offered preclinical proof of principle of small molecule LLU-206 as a novel inhibitor of ARfl/ARv7 in inherent and acquired ENZR in PCa.

Herein, we extensively screened 197 compounds from a library of novel series of thiohydantoin derivatives. The design and synthesis strategy was to select novel AR inhibitors using the second-generation AR antagonist ENZ as an initial chemical scaffold and replace the benzenic ring with a pyridine ring as the parent nucleus structure. The optimized molecular structure of LLU-206 was found to have a high binding affinity for ARfl. We systematically studied the pharmacological effects on the anti-tumor activity of LLU-206 via cell viability and tumor growth assays. LLU-206 substantially inhibited cell proliferation and tumor growth in 22RV1, LNCaP and LNCaP-ENZR cells.

Meanwhile, lower IC_{50} values and higher tumor inhibition rates further suggested better efficacy of LLU-206 against PCa cells growth than ENZ. Importantly, besides its influence on ARfl degradation, LLU-206 also affected ARv7 protein stability and transcriptional activity. LLU-206 is thus believed to be highly efficient for the treatment of CRPC and ENZR subjects.

An increasing body of evidence suggests ARv7 can act as a biomarker with predictive value for response to next-generation androgen targeting therapy in CRPC. In contrast with the ARfl structure, ARv7 lacks a hinge and LBD region; however, it contains a DNA binding domain (DBD) and an amino-terminal transactivation domain. ARv7 has been acknowledged to play an increasingly important role in driving castration-resistant growth, requiring heterodimerization with ARfl to reduce transcription of specific ARfl/ARv7 target genes and suppress the expression of growth-suppressive genes. ARv7 thus restores AR signaling, which triggers therapeutic resistance in the absence of androgens. Indeed, targeting ARfl/ARv7 heterodimerization and studying the interaction between ARv7 and ARfl are important to develop novel strategies to tackle ENZR. In this study, we identified a lead compound, LLU-206, that potently disrupted the expression of both the ARfl and ARv7 in vitro and in vivo. Furthermore, LLU-206 exhibited excellent efficiency in overcoming ARfl/ARv7-driven ENZR in PCa by disrupting ARfl/ARv7 heterodimerization. In a nutshell, LLU-206 is a promising candidate to solve ENZR in CRPC patients.

Moreover, we explored the mechanisms of LLU-206-mediated ARfl/ARv7 degradation. LLU-206 was found to promote ARfl interaction with MDM2 and ARv7, leading to degradation and ubiquitination of ARfl and ARv7 by the ubiquitin-proteasomal system. Interestingly, targeted protein degradation is a novel therapeutic concept based on drugs that destabilize proteins by promoting the binding of proteins with E3 ubiquitin ligases. Notably, MDM2 is an E3 ubiquitin ligase that interacts specifically with ubiquitin and ARfl in the final ubiquitination step, eventually triggering the ubiquitin-proteasomal system that accelerates protein degradation. In our study, the molecular docking prediction showed that LLU-206 and MDM2 were tightly bound.

Furthermore, IP analysis revealed that LLU-206 enhanced the interaction between ARfl and MDM2 or ARv7. These results suggest that LLU-206 may function as a molecular glue, which can stabilize and ubiquitinate ARfl and ARv7.
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degrade proteins (including ARv7) by orchestrating direct interactions between target and ligase. In addition, ARV7 functioning requires heterodimerization with ARII by the interaction of the ARII C-terminal with the N-terminal FxxLF motif in ARv7 and by the DNA-binding domain of ARII or ARv7. Dimerization is an absolute and indispensable requirement for constitutive ARV7 DNA-binding and transcriptional activation function. Surprisingly, in the present study, LLL-206 significantly inhibited the formation of dimers and downstream transcriptional regulation of ARII and ARv7, which may be related to AR ubiquitination and degradation. ARV7 repressor genes were upregulated in LNCaP ENZR cells (compared to LNCaP cells). This may be a dynamic change caused by compensatory responses and heterogeneity after drug resistance. The formation of dimers involves the connection of the DBD–DBD and NTD–LBD regions. In our previous studies, we found that LLL-206 could compete with AR–LBD to block the formation of dimers. Whether LLL-206 binds to the NTD or DBD regions requires more in-depth research. In addition, the mechanisms underlying ARV7 regulation by LLU-206 and how it promotes interactions between ARv7 and MDM2 remains undetermined in our work.

Furthermore, we evaluated the drug-like properties of LLL-206, including solubility, pharmacokinetics, bioavailability and toxicity. Common adverse effects of ENZ therapy usually include neurotoxicity and neurologic symptoms such as headache or seizures. Therefore, it is of paramount importance to monitor drug levels in the brain. Interestingly, we found that ENZ could cross the blood–brain barrier and accumulate within the brain, while no LLL-206 was found in the mice brain. Furthermore, the acute toxicity tests showed that treatment with a high dose (1200 mg/kg) of LLL-206 induced no significant vital organ injury, suggesting that LLL-206 might be a safer drug candidate neurotoxicity than ENZ-based therapy. However, major concerns, including solubility and bioavailability, were encountered with LLL-206 as the drug base. Multiple attempts were made with dissolving solvents, including cyclodextrin inclusion, solid dispersion, surfactant solubilization and nanocrystalline suspension, but to no avail. Ultimately, to achieve a similar drug exposure, 25 mg/kg LLL-206 (via a tail vein injection), was selected, which is 2.5 times higher than the dose of ENZ used (10 mg/kg, p.o.) (Supporting Information Table S2). Therefore, although LLL-206 has exhibited significant practical value and potential for preclinical research, further studies are essential to increase the robustness of our findings and improve the bioavailability of LLL-206.

5. Conclusions

We screened and characterized LLL-206 as a novel compound with excellent drug-like characteristics that can significantly inhibit tumor growth and overcome ENZR in PCa cell lines. Our experimental data further confirmed that the pharmacological effects of LLL-206 might be attributed to the degradation of ARII/ARv7 via the MDM2 dependent ubiquitin–proteasome system, possibly by interfering with ARII and ARV7 interaction. Accordingly, LLL-206 can be considered a new potential drug candidate for the treatment of PCa, CRPC and ENZR CRPC.

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Author contributions

Yan Li performed experiments, organized data and wrote the original manuscript. Chen Wang, Zhen Chen and Guangjiang Shi revised the manuscript. Yan Li, Ya Chu, Xiaobin Wang, Wanjie Ye, Yuhong Han, Chun Shan, Dajia Wang, Zhihui Zhao, Shijia Du and Shuqian Ma carried out the experiments. Wei He improved the dosage form of LLL-206. Jingwei Jiang performed molecular docking. Di Zhang and Xi Xu synthesized compounds. Zhiyu Li, Yong Yang and Hongxi Wu designed experiments and revised 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.2022.05.003.

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