Targeting adenocarcinoma and enzalutamide-resistant prostate cancer using the novel anti-androgen inhibitor ADA-308

SHAGHAYEGH NOURUZI1,2*, FRASER JOHNSON1*, SAHIL KUMAR1, OLENA SIVAK1, NAKISA TABRIZIAN1,2, MILLA KOISTINAH03, ANU MUONA3 and AMINA ZOUBEIDI1,2

1Vancouver Prostate Centre, Vancouver, BC V6H 3Z6, Canada; 2Department of Urology, Faculty of Medicine, University of British Columbia, Vancouver, BC V5Z 1M9, Canada; 3Aranda Pharma Ltd., 70210 Kuopio, Finland

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Abstract. Prostate cancer (PCa) is the leading cause of cancer-related death among men worldwide. PCa often develops resistance to standard androgen deprivation therapy and androgen receptor (AR) pathway inhibitors, such as enzalutamide (ENZ). Therefore, there is an urgent need to develop novel therapeutic strategies for this disease. The efficacy of ADA-308 was evaluated through in vitro assessments of AR activity and cell proliferation, alongside in vivo studies. ADA-308 has emerged as a promising candidate, demonstrating potent inhibition of AR-sensitive adenocarcinoma as well as ENZ-resistant PCa cell lines. The results of the study revealed that ADA-308 effectively blocked AR activity, including its nuclear localization, and inhibited cell proliferation in vitro. Furthermore, ADA-308 demonstrated notable efficacy in vivo, with a robust antitumor response in ENZ-resistant models. These findings establish the role of ADA-308 as a potent AR inhibitor that overcomes resistance to AR-targeted therapies and highlights its potential as a novel therapeutic approach in advanced PCa management.

Introduction

Prostate cancer (PCa) is the second most common cancer among men (1), and remains a major health challenge owing to it rising incidence and mortality rates (2). According to the World Health Organization, PCa is the fourth most common cancer globally, with ~1.4 million new cases and ~375,000 deaths reported in 2020 alone (3), emphasizing the urgent need for improved prevention and diagnostic strategies. PCa progression is highly dependent on the androgen receptor (AR), which fuels tumor growth and survival (4). The mainstream treatment for localized and metastatic PCa is androgen deprivation therapy (ADT), which reduces circulating androgens and abrogates AR signalling to prevent disease progression (5,6). Despite treatment with ADT, AR signalling is re-activated in most patients and these patients evade therapy-induced castration conditions, resulting in the recurrence of PCa as castration-resistant PCa (CRPC) (7,8). Re-activation of AR signalling occurs despite low levels of androgens in CRPC. Thus, the AR plays a central role in mediating tumour survival. Treatment with second-generation androgen receptor pathway inhibitors (ARPIs) such as enzalutamide (ENZ; also known as MDV3100), abiraterone and apalutamide has been successful in managing CRPC tumors and increasing patient survival (9,10). The competitive non-steroidal AR antagonist, ENZ, improves survival in patients with non-metastatic CRPC (11,12). Despite its potent AR pathway inhibition, the benefits of ENZ are short-lived, and patients inevitably progress to metastatic CRPC (mCRPC) (13,14). ARPI resistance represents a clinical challenge due to the lack of third-line treatment options. Taken together, these findings highlight the urgent need for new therapeutic options for refractory patients with mCRPC, including those resistant to second-generation ARPIs.

The progression of CRPC to ARPI resistance may be mediated through adaptive responses that activate AR-signaling via other pathways. A number of underlying mechanisms exist, including the alteration of AR signalling via i) aberrant glucocorticoid receptor upregulation (15), ii) AR splice variants (such as AR-V7) (16,17), iii) AR gene mutations (18), iv) an increase in AR expression (19), and v) enhancer amplification and duplication of the AR gene (20-22). CRPC predominantly remains AR+ (23,24), and a subset of ENZ-resistant models display AR reactivation (25,26), which demonstrates the importance of AR signalling in mCRPC and indicates that it remains a therapeutic vulnerability.

In the present study, the efficacy of ADA-308 was explored, as a possible rigorous benchmark against established anti-androgens, such as ENZ and darolutamide (ODM-201) (27). The mechanism of action of ADA-308 was investigated, particularly in terms of its AR inhibition activity in both AR-sensitive
adenocarcinoma (Adeno) and ENZ-resistant cell models. Furthermore, the ability of ADA-308 to inhibit AR nuclear translocation and its impact on proliferation in vitro and on tumor growth in vivo were examined to establish its potential as an anti-androgen therapeutic option.

Materials and methods

**Compound.** ADA-308 was synthesized by Aranda Pharma Ltd. and manufactured by Jubilant Chemsys Ltd. ADA-308 boasts a notable purity level of 99.8%, signifying its high quality and consistency. The batch no. J763-Z01220-083 identifies the compound used in the present study.

**Cell lines and cell culture treatments.** Cell lines and cell culture treatments were maintained under standard conditions of 37°C and 5% CO₂. The LNCaP cell line was obtained from ATCC. 49C<sub>ENZR</sub> (49C enzalutamide-resistant) and 49F<sub>ENZR</sub> cultured in 10 µmol/l ENZ. In addition, when indicated, cell lines were treated with 10 µmol/l ENZ or 10 µmol/l Inc.; cat. no. A3160701). ENZ-resistant cell lines were also supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 11875093) supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.; cat. no. A3160701). ENZ-resistant cell lines were also cultured in 10 µmol/l ENZ. In addition, when indicated, cell lines were treated with 10 µmol/l ENZ or 10 µmol/l ADA-308 (Aranda Pharma Ltd.). For hormone stimulation with synthetic androgen, cells were treated with 10 nM R1881 (MilliporeSigma; cat. no. 965-93-5).

**In vivo study.** The animal experiments adhered to protocols approved by The Animal Care Committee at The University of British Columbia (Vancouver, Canada; approval no. A16-0246; approval date, 12/15/2016). Mice were housed in ventilated cages (4 mice per cage) under controlled conditions, including constant humidity (25-27%) and temperature (21-22°C), with a 12-h light-dark cycle. The mice were provided with unrestricted access to rodent Chow diet and water and experiments on the mice began between 6-8 weeks of age. At the experimental or humane endpoint, mice were euthanized using an inhalant anesthetic (3% isoflurane) followed by carbon dioxide (50% of the cage volume per min). A secondary accepted physical method of euthanasia (decapitation) was performed to prevent revivification. For castration, 2.5% isoflurane vaporizer and 2 l/min oxygen were used for anesthesia, providing both the induction and maintenance doses. A total of 12 mice were assigned per treatment group. The mice weighed ~20 g at the start of the study and were supplied by Envigo.

Male athymic mice were castrated and allowed to recover from surgery for 3 days. Then, 2x10⁶ 49F<sub>ENZR</sub> cells were inoculated twice, once per site on the right and left flanks for the first in vivo study using 25 or 50 mg/kg ADA-308 and once on the right site for the second study using 12.5 or 25 mg/kg ADA-308. The mice were recovered for 3 days then administered 10 mg/kg ENZ daily until the tumor volume reached 200 mm³. Next, ENZ (10 mg/kg) was either continued (ENZ group) or switched to vehicle, ADA-308 at 12.5, 25 or 50 mg/kg twice a day (BID), or ODM-201 (Orion Pharmaceuticals Corporation) at 50 mg/kg BID. All treatments were administered orally (gavage) and all in vitro studies utilized a common vehicle, a 2% Tween-0.5% carboxymethyl cellulose sodium salt solution. The tumor volumes were measured three times per week in a blinded fashion and calculated using the formula:

\[ \text{Volume} = \frac{\pi \times (\text{length} \times \text{width} \times \text{height})}{6} \]

Recruitment was conducted in 10 cycles, the length of ENZ treatment ranging from 19 to 52 days. Mice were sacrificed at predetermined time points after treatment, when the tumor volume reached 2,000 mm³, when tumors reached >10% of the body weight or the body weight loss was >15%, whichever came first. The maximum long diameter of a single tumor was 21 mm and the maximum sum of the long diameter of both the left and right tumors in a single mouse was 33.5 mm. The maximum sum of the tumor volume of both the left and right tumors in a single mouse was 2,425 mm³. While the humane endpoint was set at a tumor volume of 2,000 mm³, the individual mouse in question that exceeded the tumor size belonged to the ENZ treatment group and had a tumor volume of 1,462 mm³ at the 2.5-week time point. Therefore, the mouse was not sacrificed at that time. By the 3-week time point, when tumors were measured again, the tumor volume had grown beyond the endpoint, resulting in a measurement of 2,425 mm³, at which point the mouse was then sacrificed.

**Western blotting.** Proteins were extracted from cells cultured in vitro. The cells were washed once with 1X PBS and subsequently lysed using RIPA buffer (Thermo Fisher Scientific, Inc.; cat. no. PB9901) enriched with a 1X concentration of cOmplete EDTA-free protease inhibitors cocktail (Roche Diagnostics; cat. no. 1183617001) and phosphatase inhibitors (PhosSTOP; Roche Diagnostics; cat. no. 4906845001). Following protein quantification using the BCA protein assay (Thermo Fisher Scientific, Inc.; cat. no. 23225), the samples were subjected to a 5-min boiling step in 4X SDS sample buffer. The 4X SDS sample buffer contained 8-10% SDS, 200 mM Tris-HCl (pH 6.8), 40% glycerol, 0.02% Bromophenol Blue and 5% β-mercaptoethanol. Equal amounts of protein (40 µg per lane) were resolved by SDS-PAGE using 10% polyacrylamide gels. The proteins were transferred onto PVDF membranes, then the membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences; cat. no. 15590545) at room temperature for 30 min and probed with primary antibodies at the specified dilutions overnight at 4°C. The membranes were washed three times with 1X TBST (2% Tween-20) for 10 min, then probed with the appropriate secondary antibody for 1 h at room temperature. The membranes were washed three times with 1X TBST for 10 min before visualization using a LI-COR Odyssey Scanner. The immunoblotting utilized the following antibodies: AR (clone D6F11; 1:1,000; Cell Signaling Technology, Inc.; cat. no. S153) and prostate-specific antigen (PSA; clone D6B1; 1:5,000; Cell Signaling Technology, Inc.; cat. no. 5365), with Vinculin (clone hvin-1; 1:25,000; Cell Signaling Technology, Inc.; cat no. 4650) serving as the loading control. The secondary antibodies included IRDye 800CW donkey anti-rabbit (1:10,000; LI-COR Biosciences; cat. no. 926-32213). Uncropped western blots are shown in Fig. S1.

**Reverse transcription-quantitative PCR (RT-qPCR).** Cells were plated in 100-mm plates at a density of 4.0x10⁶ cells/plate in RPMI media supplemented with 10% FBS and...
1% penicillin/streptomycin (pen/strep). The following day, cells were treated with either vehicle (DMSO, final concentration 0.1%), ENZ (10 µM) or ADA-308 (concentrations of 1, 2, 5, 7.5 or 10 µM; final concentration in 10 ml). After 72 h of treatment, the cells were washed with 1X PBS and detached in PBS/5 mM EDTA/sodium vanadate, pelleted (centrifuged at 1,200 x g for 5 min at 4°C) and resuspended in TRIzol (Thermo Fisher Scientific, Inc.; cat. no. 15596026) for RNA extraction. For reverse transcription, cDNA synthesis was performed using SuperScript™ IV Reverse Transcriptase (SSIV RT; Thermo Fisher Scientific, Inc.; cat. no. 18090010), according to the manufacturer's protocol. Briefly, 0.2 µg RNA was mixed with oligo(dT)20 primers (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. 18418020) and dNTPs (Invitrogen; Thermo Fisher Scientific, Inc. cat. no. 10297018), and the mixture was incubated at 65°C for 5 min. After chilling on ice, the buffer, DTT and SuperScript IV enzyme were added. The reaction was carried out at 23°C for 10 min, followed by 50°C for 30 min, and terminated at 80°C for 10 min. cDNA was then used for qPCR analysis using SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Inc.; cat. no. 4309155), according to the manufacturer's instructions. Primers and cDNA templates were added to 38-well plates in triplicate. The expression of each gene was normalized to the expression of GAPDH and the 2^−ΔΔCq method (29) was used to quantify the change in expression from vehicle (DMSO) treatment. Experiments were repeated twice and the mean ± SEM of the independent experiments are shown. The primer sequences were as follows: GAPDH forward, GGAGGCGAGATCCTCTCAAAT; GAPDH reverse, GGCCTGTGTCACTACTTCT CATTG; PSA/kallikrein-3 (KLK3) forward, CACAGGCTGT TTTCATCTGTA; KLK3 reverse, AGGTCCATGACCCTTC ACAG; homeobox protein Nkx-3.1 (NKX3.1) forward, GGA CTGAGTGAGCCCTTGG; NKX3.1 reverse, CAGCCTA GATTTCCTCCTGTC; FK506 binding protein 5 (FKBP5) forward, TCCCCCTGAAATGCACTCT; FKBP5 reverse, GCCACATCTCTGAGCTCAA; transmembrane protease, serine 2 (TMPRSS2) forward, TGGTAGTGTCTCCCCAG CCTAC; TMPRSS2 reverse, AAAACGACGTGAATAGGCC CA; AR forward, TACCAAGCTCAAGACGCT; and AR reverse, GCTTCACTGGTGTTGGAAT. The temperature protocol used for the qPCR reaction was as follows: Initial Denaturation at 95°C for 10 min; amplification cycles (35 cycles): Denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec; final extension at 72°C for 5 min.

Microscopy. Cells were plated in RPMI media supplemented with 5% charcoal-stripped serum (Thermo Fisher Scientific, Inc.; cat. no. A3382101) on poly-L-lysine-coated coverslips at a density of 1x10^5 cells/well. The following day, the cells were pretreated with ENZ (10 µM), ADA-308 (10 µM) or DMSO (0.1%) for 24 h. Then, the cells were treated with either DMSO or the AR agonist, R1881, at a concentration of 10 nM for 20 min. The cells were fixed with 100% ice-cold methanol for 10 min, followed by 1X PBS washes. The cells were then incubated with anti-AR antibody (1:1,000; clone 441; Santa Cruz Biotechnology, Inc.; cat. no. sc-7305) for 1.5 h at room temperature, followed by washes with 1X PBS to remove unbound antibodies. Next, the cells were incubated with a secondary anti-mouse Alexa 488-conjugated antibody (1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A21202) for 45 min at room temperature. The cells were washed with 1X PBS to remove unbound secondary antibodies, then DAPI (Thermo Fisher Scientific, Inc.; cat. no. D1306) coverslips were mounted on slides to stain the nucleus. Fluorescent images were collected using a x60 oil immersion objective, FV3000RS confocal microscope equipment and Olympus FV31S-SW software (version 2.3.2.169; Olympus Corporation).

Cell proliferation. Cells were seeded at a density of 2,000 cells per well in 96-well plates in RPMI-1640 media supplemented with 5% FBS and treated with either vehicle [DMSO (0.1%), ENZ (10 µM), ADA-308 (10 µM) or ODM-201 (10 µM)]. Each treatment condition was set up in 8 wells. The plates were placed in the IncuCyte live-cell analysis system (Essen Bioscience), and images were acquired every 12 h for 7 days. IncuCyte software (v2020C; Essen Bioscience) was used to analyze the cell confluency automatically over time.

Cell cycle. Cells were plated in 100-mm plates at a density of 2x10^5 cells/plate in RPMI media supplemented with 10% FBS and 1% pen/strep. The following day, the cells were treated with either vehicle (DMSO at 0.1%), ENZ (10 µM) or ADA-380 (concentrations of 1, 2, 5, 7.5 or 10 µM; final concentration in 10 ml). After 72 h of treatment, the cells were washed with 1X PBS and detached in PBS/5 mM EDTA/sodium vanadate, pelleted by centrifuging at 1,200 x g for 5 min at 4°C, fixed and permeabilized in 70% ice-cold ethanol for 30 min and then stored at -30°C for a minimum of 24 h. The cells were pelleted by centrifuging at 1,200 x g for 10 min at 4°C and washed in PBS, then stained in propidium iodide (PI; MilliporeSigma; cat. no. P4864) solution (50 µg/ml PI, 0.1 mg/ml RNase, 0.05% Triton X-100, 1X PBS) for 40 min at 37°C. Finally, the cells were washed and stained before flow cytometry analysis. Data were acquired by FACS on a Canti II (BD Biosciences). Data were analyzed using FlowJo software (version 10.4.2; FlowJo LLC). Representative histograms are shown in Fig. S2.

Luciferase assay. Cells were seeded in 12-well plates at a density of 1x10^5 cells/well in RPMI media supplemented with 10% FBS and 1% pen/strep. The following day, the cells were transfected with 0.2 µg of the probasin RR3 luciferase reporter using TransIT-2020 (Mirus Bio, LLC) in Opti-MEM media (Gibco; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The Probasin ARR3 tk-luc reporter was kindly provided by Dr Martin Gleave's Lab at Vancouver Prostate Centre (Vancouver, Canada) (30). After 24 h, the Opti-MEM transfection mix was removed and replaced with 10 µM (final) of the compound (ENZ, ODM-201 or ADA-308) in RPMI media supplemented with 10% FBS and 1% pen/strep in triplicate. The following day, the wells were washed once with pre-warmed 1X PBS, then incubated with 200 µl of 1X Passive lysis buffer (Promega Corporation; supplier no. E1941; cat. no. PAE1941) at room temperature with shaking for 30 min and frozen at -80°C for 45 min. The plates were thawed, the lysate was collected in microcentrifuge tubes and the debris was cleared via centrifugation at 1,200 x g for 10 min at 4°C. Next, 50 µl (per well) of the supernatant was
added to 96-well white, flat bottom plates, then 75 µl luciferase assay buffer (Promega Corporation; cat. no. E1910) was automatically injected per well. After 30 sec of incubation, signal was detected by a luminescent plate reader (Tecan infinite M200Pro). Fluorescence units were normalized to the protein concentration per sample (BCA assay) and calculated relative to the control condition [DMSO for LNCaP; ENZ (10 µM)] or ADA-308 (10 µM). Total RNA was isolated from the cells after 72 h of treatment using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Inc.). The library was generated using the NEBnext Ultra ii Stranded RNA Library Prep Kit (New England BioLabs, Inc.; cat. E7770S), the quality of the RNA samples was assessed by measuring the 230/260 and 260/280 ratio using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.), ensuring values were >1.8 and 2, respectively. Sequencing was performed on an Illumina NextSeq 500 (42x42-bp paired-end reads) by the University of British Columbia Sequencing + Bioinformatics Consortium (Vancouver, Canada), targeting 20 million reads per sample. Data was de-multiplexed using bcl2fastq2 Conversion Software (version 2.20; Illumina, Inc.), and read sequences were aligned to the human reference genome, hg38, using STAR aligner (version 2.7.8a) (31). Assembly and differential expression were estimated using Cufflinks software (version 2.2.1) (32), available through the Illumina BaseSpace Sequence Hub. Gene expression data (raw count data) were normalized using ‘DESeq’ (33) in Rstudio (version 4.1.2; https://cran.r-project.org/), and subsequently, log2 was transformed. Unsupervised clustering was generated using R, and data were visualized using the R ‘ggplot’ program or GraphPad Prism (version 8; Dotmatics). The significance of the expression level differences between the treatment samples was determined using an unpaired t-test in GraphPad Prism or R.

Chromatin immunoprecipitation-sequencing (ChIP-seq) data analysis. ChIP-seq Fastq files were downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). Publicly available AR ChIP-seq datasets [GSM1069669 and GSM1236925, from Chen et al (34)] used in the present study were downloaded from GSE43791. Publicly available control LNCaP RNA-seq [GSM4777223 and GSM4777224, from Davies et al (26)] data were downloaded from GSE138460. Data was processed using FastQC (version 0.11.9) (35) for quality control analysis. Adapter sequences were removed using Cutadapt (version 1.18; https://cutadapt.readthedocs.io/en/stable/) and reads were aligned to the human genome reference, hg38, using BWA-MEM software (version 0.7.17) (36). SAM files were converted to BAM files using SAMtools software (version 1.1.2) (37). MACS2 (version 2.2.7.1) (38) was used to call peaks with a false discovery rate (FDR) of 0.05 using the narrow peak caller for AR-bound genes. DeepTools (version 2.30.0) (39) was used to visualize data, and BEDtools (version 2.28.0) (40) generated shared and unique peaks between dihydrotestosterone (DHT) and ENZ AR ChIP-seq samples. The peaks were annotated using HOMER (version 3.0; http://homer.ucsd.edu/homer/).

Gene ontology and pathway analysis. Pathway analysis was conducted using gene set enrichment analysis (GSEA) software, available from the Broad Institute (Massachusetts Institute of Technology) and gProfiler (41). This analysis aimed to discern the functions associated with differentially expressed genes within the Molecular Signatures Database (version 7.1) (42). The GSEA tool was utilized in classic mode to identify significantly enriched biological pathways. Pathways exhibiting enrichment with a nominal P<0.05 and FDR <0.25 were considered statistically significant. For single-sample GSEA, gProfiler, a web server designed for functional enrichment analysis and the conversion of gene lists, was utilized.

Statistical analysis. Statistical analysis was conducted using Microsoft Excel and GraphPad Prism software (version 8; GraphPad). All presented in vitro experiments were independently replicated a minimum of three times. To analyze data variance between multiple groups, a one-way ANOVA followed by the Dunnet’s test were used to perform multiple comparisons within each group compared with the control group, which is depicted in bar charts. For longitudinal profiling experiments, a two-tailed unpaired Student’s t-test was performed to determine the statistical difference at the final time point. Visualization was performed using GraphPad Prism 8 or Rstudio (version 4.1.2; https://cran.r-project.org/). P<0.05 was considered to indicate a statistically significant difference.

Results

Introduction to ADA-308. ADA-308, a novel arylamide compound, emerged following an extensive and meticulous design process involving the synthesis and evaluation of over 650 carefully crafted structures, comprising >220 profiled small molecules and various chemical scaffolds. In preliminary pharmacokinetic studies (unpublished data), ADA-308 exhibited notable characteristics in mice. When administered orally at 100 mg/kg in mice, ADA-308 displayed a plasma half-life (T1/2) of 10.9 h. In comparison, oral administration of ADA-308 at a 30 mg/kg dose in rats resulted in a plasma T1/2 of 12.6 h. ADA-308 was designed to address treatment resistance observed with other AR antagonists. The compound was designed considering optimized binding affinity to the AR, improved pharmacokinetic properties and its ability to retain AR antagonism in CRPC cells (such as when AR is upregulated or mutated). In addition to ENZ-resistant conditions (such as F876L AR mutation), ADA-308 was screened in a panel of CRPC AR mutants (T877A and W741L), where it retained its antagonistic activity, highlighting its potential as a versatile and practical treatment option.

ADA-308 suppresses AR transcriptional activity in Adeno and ENZ-resistant cell lines. To assess the impact of ADA-308 treatment on AR signalling, the prostate Adeno cell line, LNCaP, was treated with ADA-308. A dose-dependent decrease in the expression of PSA, a canonical AR target, was observed, which was similar to that observed following ENZ
treatment (Fig. 1A). The mRNA expression of other canonical AR targets also decreased in a dose-dependent manner, comparable to ENZ (Fig. 1B and Table SI). Furthermore, using an androgen-responsive luciferase reporter linked to the probasin (43) promoter, known for its robust AR-specific and tissue-specific regulation (43), it was observed that ADA-308 significantly and efficiently reduced AR activity. This was similar to the results achieved with ENZ or ODM-201 (high-affinity AR antagonists) (27) (Fig. 1C).

CRPC is often treated with the potent AR antagonist, ENZ, which frequently leads to ENZ resistance through the re-activation of the AR signalling axis. To model ENZ-resistant disease, with re-activation of AR signalling, our lab previously generated ENZ-resistant cell lines, 49CENZR and 49F ENZR, by serially passaging the PCa Adeno cell line, LNCaP, in castrated mice treated with ENZ (28). These cell lines are derived from PSA+ tumors and retain PSA expression (44). These cell lines harbour the AR F876L activating mutation (45), a rare mutation in the early stages of the disease that is frequently observed in CRPC (46) and ENZ-resistant tumors (47,48). By altering the ligand binding pocket of AR, F876L allows other steroid hormones (such as corticosteroids and anti-androgens) to activate AR (49), rendering ENZ an agonist that drives phenotypic resistance (47,48). To explore the potential of re-targeting AR signaling in these models, the effect of ADA-308 on AR-dependent genes was investigated. The findings revealed that treatment with ADA-308 exhibited a dose-dependent reduction in PSA expression in both 49CENZR and 49FENZR (Figs. 1D and S3A). This reduction in AR activity was reflected by decreased mRNA expression of canonical AR target genes (Figs. 1E, S3B, and Table SI) and a significant decrease in probasin luciferase activity (Figs. 1F and S3C). Notably, treatment of LNCaP with either ENZ, ADA-308 or ODM-201 resulted in a reduction in the PSA mRNA level (Fig. S3D), with no differences observed between the different compounds. Taken together, these data demonstrated that ADA-308 acts as an AR signaling inhibitor in Adeno, particularly in ENZ-resistant cell line models.

ADA-308 inhibits AR nuclear localization in Adeno and ENZ-resistant prostate cancer cell lines. AR, a nuclear transcription factor that belong to the steroid hormone receptor superfamily (50), is activated upon binding of androgens (51). In the absence of a ligand, AR primarily resides in the cytoplasm and often form...
complexes with heat shock protein chaperones (52). However, in the presence of ligands, AR undergo homodimerization, translocates to the nucleus and attaches to androgen response elements to initiate transcription (53).

Notably, in the Adeno cell line, LNCaP, and the ENZ-resistant cell lines, 49C ENZR and 49F ENZR, AR was predominantly localized to the cytoplasm. However, AR translocates to the nucleus upon stimulation with synthetic androgen (R1881). Immunofluorescence microscopy showed that treatment with ADA-308 inhibited AR nuclear translocation in the LNCaP cell line, similar to the effect observed for ENZ (Fig. 2A and B), suggesting that both ADA-308 and ENZ effectively hindered the androgen-induced nuclear translocation of AR. In addition, AR was observed in the cytoplasm and nucleus of the ENZ-resistant cell lines, 49F ENZR. However, treatment with R1881 increased the ratio of nuclear AR to cytoplasmic AR (Fig. 2C and D). Moreover, ADA-308 treatment resulted in a modest increase in G0/G1 arrest; a ~9% increase in the G0/G1 cell population was observed, which was comparable to the ~8% increase following ENZ treatment (Figs. 3C and S4). Next, the effect of ADA-308 on ENZ-resistant cell lines was evaluated. Similar to the observations in the Adeno cell line, ADA-308 markedly prevented the androgen-induced nuclear translocation of AR, whereas ENZ treatment failed to do so (Figs. 2C, 2D and S4A). These results suggested that, following the development of ENZ resistance, ADA-308 exerted its inhibitory effect on AR signaling by preventing AR nuclear translocation. These data highlight the potential of ADA-308 as an antagonist of mutated AR and warrant further investigation into its clinical response, particularly in ENZ-resistant tumors.

**ADA-308 inhibits proliferation in vitro**. Next, the anti-proliferative properties of ADA-308 and its impact on the cell cycle were assessed. A reduction in the proliferation rate of LNCaP cells was observed upon treatment with either ADA-308 or ENZ, with ADA-308 exhibiting a more pronounced suppression of cell proliferation than ENZ (Fig. 3A). Moreover, ADA-308 treatment resulted in a modest increase in G0/G1 arrest; a ~9% increase in the G0/G1 cell population was observed, which was comparable to the ~8% increase following ENZ treatment (Figs. 3C, S4). However, it was observed that ODM-201 had a more profound effect on the cell proliferation (Fig. S4B). In addition, a significant increase in the G0/G1 cell population in the ENZ-resistant cells after ADA-308 treatment was observed (Figs. 3D, S4C).
To understand the biological impact of ADA-308, LNCaP cells were treated with ADA-308 and RNA-seq was performed. First, the changes in AR signalling induced by ADA-308 treatment were assessed. A notable inhibition of AR activity was observed as evidenced by a marked reduction in the expression of canonical AR targets (Fig. 3E). GSEA was performed to identify a range of pathways altered by ADA-308 treatment. As expected, the downregulation of AR-regulated pathways following ADA-308 treatment was observed. Notably, ADA-308 significantly inhibited pathways crucial for cell proliferation and cycle progression (Fig. 3F). These observations highlighted the promising anti-proliferative properties of ADA-308 in Adeno in vitro, particularly in the context of ENZ-resistance models that are resistant to existing treatments.

ADA-308 modulates AR-bound target genes and associated pathways. To evaluate the efficacy of ADA-308 in comparison with ENZ, RNA-seq of LNCaP cells treated with either ADA-308 or ENZ was conducted. Unsupervised clustering revealed that the treated samples clustered together and were distinct from those of the control group (Fig. 4A). Although a significant number of differentially expressed genes after treatment with these AR inhibitors was observed (Fig. 4B), the difference between ADA-308 and ENZ was not statistically significant (Fig. 4C). Subsequent analysis identified 1,081
genes that were downregulated by both ADA-308 and ENZ treatments (Fig. 4D). GSEA revealed that these commonly downregulated genes were associated with pathways regulating the cell cycle, proliferation and the androgen response (Fig. 4E and Table SII). This was consistent with our previous findings demonstrating that ENZ treatment led to reduced proliferation.
and induced G0/G1 arrest in LNCaP cell lines (54,55), but notably highlighting the effectiveness of ADA-308 in the context of ENZ-resistant models where ENZ was less responsive (Fig. 3A-D).

To delve deeper, publicly available AR ChIP-seq data were leveraged (34). The regions bound by the AR in the presence of DHT or ENZ were examined. Notably, a substantial number of AR-bound regions (34,682 peaks) were lost following ENZ treatment (Fig. 4F), consistent with previous reports indicating that ENZ reduces AR chromatin binding and nuclear localization (56) and in alignment with aforementioned observations (Fig. 2A and B). However, despite ENZ treatment, ~15,000 regions remained bound by AR. The AR-bound regions that were lost following ENZ treatment were specifically focused on, and 12,382 genes within this region were identified (Fig. 4F). Following integration of the RNA-seq data, a significant association between ADA-308 and ENZ-regulated AR target genes was revealed (Fig. 4G). Moreover, the GSEA results shed light on the functional consequences of these alterations, with AR-bound genes upregulated following treatment associated with stemness, whereas downregulated genes were linked to androgen signaling, luminal phenotype
and proliferation (Fig. 4H). Collectively, these data suggested that ADA-308 exerted effects comparable to those of ENZ in modulating critical AR-bound target genes and their associated pathways.

**ADA-308 reduces tumor growth in vivo.** Investigation into the effects of ADA-308 revealed its ability to inhibit cell proliferation in vitro. To assess the in vivo pharmacodynamics activity of ADA-308, castrated mice harboring 49F\(^{\text{ENZ}}\) ENZ-resistant xenograft tumors were treated with ADA-308, ENZ or ODM-201 for comparison. For this, male athymic mice were castrated and allowed to recover from surgery. Then, ENZ-resistant 49F\(^{\text{ENZ}}\) cells were inoculated, and mice were administered ENZ daily until the tumor volume reached 200 mm\(^3\). Thereafter, the treatment regimens were adjusted, with ENZ either continued or replaced with vehicle, ADA-308 at 25 mg/kg BID, ADA-308 at 50 mg/kg BID or ODM-201 at 50 mg/kg BID (Fig. 5A-C and Table SIII). The mice were treated for up to 8 weeks or until the tumor volume reached 1,500 mm\(^3\).

The reduction in proliferation rates observed in vitro translated into a notable in vivo antitumor response. It was observed that both doses of ADA-308 (25 or 50 mg/kg) exhibited improved antitumor responses in the ENZ-resistant cell model compared with ENZ or ODM-201. Notably, in the ENZ-treated group, most mice reached the study endpoint by 3 weeks (Fig. 5D). The percentage change in tumor volume after treatment with ADA-308 was significantly lower (Fig. 5E and Table SIV), leading to higher survival rates (Fig. 5F and Table SIII). Notably, prior ENZ administration did not compromise the efficacy of ADA-308. In addition, testing lower doses of ADA-308 (12.5 mg/kg BID) resulted in a significantly reduced tumor volume in ENZ-resistant tumors (Fig. S5A-C). Overall, these data elucidated the in vivo efficacy of ADA-308 and its superior capacity to inhibit tumor growth in ENZ-resistant 49F\(^{\text{ENZ}}\) xenograft models.

**Discussion**

It is now understood that CRPC retains its androgen sensitivity, both in the early stages of the disease as well as following the successful treatment with next-generation ARPIs (9,57,58). This dependence on the AR for growth (59-61) highlights the continued significance of the AR as a therapeutic target in PCa (62). However, the response to second-generation ARPIs is often only temporary, and resistance poses an unavoidable challenge. As a result, several AR antagonists including ARN-509 (47) and ODM-201 (27), have been developed and evaluated for inhibition of AR activity. Therefore, development of alternative and novel AR-targeted therapies is of paramount importance.

The ADA-308 compound was originally designed to overcome the treatment resistance to other AR antagonists in advanced PCa. The present study demonstrated that ADA-308 can potentially reduce AR activity in ENZ-sensitive and ENZ-resistant preclinical models. The investigation encompassed two distinct PCa cell models: LNCaP (representing Adeno) and the ENZ-resistant 49F\(^{\text{ENZ}}\) and 49C\(^{\text{ENZ}}\) cell lines (which no longer responded to ENZ treatment). Administration of ADA-308 in these models resulted in a significant inhibition...
of AR signalling and the accumulation of cells in the G0/G1 phase of the cell cycle, a response comparable to that of ENZ in LNCaP cells. It was therefore demonstrated that ADA-308 is a very potent AR inhibitor in PCa research models including those resistant to ENZ. Mechanistically, it was shown that the mechanism of action of ADA-308 closely parallels that of ENZ and ODM-201 (Fig. 6). Notably, ADA-308 hindered androgen-induced AR nuclear localization in LNCaP cells, which is a critical step in AR activation and targeted gene transcription. In ENZ-resistant (49FENZR and 49CENZR) cell lines, ENZ significantly failed to inhibit androgen-induced AR nuclear localization, while ADA-308 prevented this effect. Moreover, comparing the effect of ADA-308 to ENZ on the transcriptome of LNCaP cells, the data revealed that ADA-308 was comparable to ENZ in suppressing genes regulated by the AR or those associated with proliferation. Notably, upon ADA-308 treatment of LNCaP cells, an increased expression of AR-bound genes associated with the stemness pathway was observed, similar to ENZ treatment. This raises a noteworthy concern regarding whether treatment with ADA-308 can induce lineage plasticity. Lineage plasticity has been postulated to contribute to the failure of ARPs in PCa, representing an established mechanism of treatment resistance associated with the loss of luminal lineage, and an induction of alternative programs including stem cell-like phenotypes (26,63-65). Therefore, it is important to evaluate whether ADA-308 induces lineage plasticity.

In the present study, ADA-308 demonstrated a superior \textit{in vitro} anti-proliferative effect compared with ENZ in ENZ-resistant cell line models. Moreover, the presented \textit{in vivo} study provided compelling evidence that ADA-308 reduced tumor growth in ENZ-resistant models. The anti-tumor effect of ADA-308 was accompanied by an increase in overall survival. Collectively, these findings suggested that ADA-308 may emerge as a promising and viable candidate for future clinical development in CRPC, particularly in an ENZ-resistant context where ENZ treatment has failed, thereby offering a viable treatment strategy in the evolving landscape of PCa therapy. Finally, a more comprehensive understanding of the safety profile, long-term effects and potential resistance mechanisms of ADA-308 is essential as we consider its transition into clinical development.

Although the present study provided valuable insights into the therapeutic potential of ADA-308 in PCa, particularly in overcoming resistance to other AR antagonists such as ENZ, there are some limitations to the findings. One limitation of the present study is the limited number of models, which may not fully represent the genetic and phenotypic diversity of PCa observed in a broader patient population. Additionally, the \textit{in vivo} studies were conducted exclusively in mouse models, which, despite their utility, cannot perfectly mimic the complex human tumor microenvironment and immune interactions. Notably, ARPs can lead to the development of lineage plasticity, a mechanism of resistance in which cells alter their lineage to acquire an alternative lineage that is often associated with stem-cell and neuronal characteristics. Therefore, future studies are needed to better characterize whether ADA-308 treatment leads to the activation of resistance mechanisms, including lineage plasticity. Additionally, longitudinal studies monitoring long-term outcomes and potential side effects are essential to ensure that ADA-308 can provide sustainable benefits for the treatment of PCa. In addition, while it was shown that ADA-308 reduced PSA expression similar to ODM-201 and that the \textit{in vivo} effects of the compounds were similar, without further investigation regarding the long-term effect of ADA-308, we cannot comment on whether ADA-308 will be a preferred option for treatment of PCa to ODM-201.

In the present study, the significant efficacy of ADA-308 in suppressing AR signalling and reducing proliferation \textit{in vitro} and \textit{in vivo} was highlighted. These findings are particularly noteworthy and relevant given the growing occurrence of resistance to potent ARPs (66-68) and the limited number of therapeutic options following the development of resistance. The ability of ADA-308 to inhibit AR activity in models that have developed resistance to ENZ suggests its potential as an effective agent to follow ARP resistance. However, while there is potential for ADA-308 in PCa, the commercialization of the program in PCa became challenging for Aranda Pharma Ltd. due to changes in clinical practice (such as the sequential use of second-generation AR inhibitors is not recommended when one fails) and high competition in the market.

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Availability of data and materials

The RNA-seq data generated in the present study may be found in the GEO database under the accession no. GSE267309 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE267309. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

SN, FJ, SK, OS, NT, MK, AM and AZ confirm the authenticity of all the raw data, conceived this study and took responsibility for the quality of the data. AM and MK contributed to the study design. AM, SN and FJ participated in the analysis and interpretation of data and prepared all figures. FJ and SK...
performed all the in vitro experiments and acquired data. NT performed the proliferation assay and assisted in the revision of this manuscript. OS performed the in vivo experiments. SN wrote the manuscript. AZ, AM and FJ reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the procedures and protocols of the Laboratory Animal Center of the University of British Columbia (Vancouver, Canada; approval no. A16-0246; approval date, 12/15/2016).

Patient consent for publication

Not applicable.

Competing interests

Aranda Pharma Ltd. owns the IP of ADA-308. AM and MK are shareholders of Aranda Pharma Ltd. All other authors declare that they have no competing interests.

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