RESEARCH

Peptides disrupting TM4SF3 interaction with AR or AR-V7 block prostate cancer cell proliferation

Prabesh Khatiwada[1,2,*], Ujjwal Rimal[1,*], Mamata Malla[1], Zhengyang Han[1] and Lirim Shemshedini[1]

[1]Department of Biological Sciences, University of Toledo, Toledo, Ohio, USA
[2]Center for Translational Immunology, Columbia University, New York, NY, USA

Correspondence should be addressed to L Shemshedini: lirim.shemshedini@utoledo.edu

*(P Khatiwada and U Rimal contributed equally to this work)

Abstract

Androgen receptor (AR) plays a vital role in the development and progression of prostate cancer from the primary stage to the usually lethal stage known as castration-resistant prostate cancer (CRPC). Constitutively active AR splice variants (AR-Vs) lacking the ligand-binding domain are partially responsible for the abnormal activation of AR and may be involved in resistance to AR-targeting drugs occurring in CRPC. There is increasing consensus on the potential of drugs targeting protein–protein interactions. Our lab has recently identified transmembrane 4 superfamily 3 (TM4SF3) as a critical interacting partner for AR and AR-V7 and mapped the minimal interaction regions. Thus, we hypothesized that these interaction domains can be used to design peptides that can disrupt the AR/TM4SF3 interaction and kill prostate cancer cells. Peptides TA1 and AT1 were designed based on the TM3SF3 or AR interaction domain, respectively. TA1 or AT1 was able to decrease AR/TM4SF3 protein interaction and protein stability. Peptide TA1 reduced the recruitment of AR and TM4SF3 to promoters of androgen-regulated genes and subsequent activation of these AR target genes. Peptides TA1 and AT1 were strongly cytotoxic to prostate cancer cells that express AR and/or AR-V7. Peptide TA1 inhibited the growth and induced apoptosis of both enzalutamide-sensitive and importantly enzalutamide-resistant prostate cancer cells. TA1 also blocked the migration and malignant transformation of prostate cancer cells. Our data clearly demonstrate that using peptides to target the important interaction AR has with TM4SF3 provides a novel method to kill enzalutamide-resistant prostate cancer cells that can potentially lead to new more effective therapy for CRPC.

Key Words

- AR
- AR-V7
- TM4SF3
- prostate cancer
- peptides

Endocrine Oncology (2023) **3**, **e230010**

Introduction

The second leading cause of cancer deaths among American men is prostate cancer (Siegel *et al.* 2020). Upon binding androgen, androgen receptor (AR) regulates the expression of target genes that lead to differentiation, proliferation, and transformation of prostate cancer cells (Simental *et al.* 1991, Gelmann 2002, Huang & Tindall 2002, Lonergan & Tindall 2011). Androgen deprivation therapy (ADT), through either physical or chemical castration, is the standard treatment of prostate cancer (Huggins *et al.* 1941, Huggins & Hodges 1972, Wasson *et al.* 1993, Denmeade and Isaacs 2002). ADT often results initially in a positive response in patients, but the disease usually relapses to the more aggressive and usually lethal form called castration-resistant prostate

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

cancer (CRPC) (van der Kwast *et al.* 1991, Wilt *et al.* 2008). CRPC remains largely dependent on AR signaling and becomes resistant to ADT via multiple mechanisms, including the expression of AR splice variants like AR-V7 that acts constitutively (Guo *et al.* 2009, Sun *et al.* 2010, Qu *et al.* 2015). Given that the current ADT drugs, including enzalutamide and abiraterone, target the androgen dependence of AR activation, they mostly fail in treating CRPC that express AR variants like AR-V7 (Haile & Sadar 2011, Wadosky & Koochekpour 2017).

Transmembrane 4 superfamily 3 (TM4SF3), also known as TSPAN8 or CO-029, belongs to the tetraspanin family (Huang *et al.* 2005). TM4SF3 was identified as a tumor-associated antigen due to the correlation of its high expression with growth, invasion, and metastasis in different carcinomas (Hemler 2014, Wei *et al.* 2015, Anami *et al.* 2016, Dong *et al.* 2016, Zhu *et al.* 2019), including pancreatic (Wang *et al.* 2013), esophageal (Zhou *et al.* 2008), gastric (Wei *et al.* 2015), and colorectal (Zhang *et al.* 2020) carcinomas. However, the role of TM4SF3 in prostate cancer was largely unknown (Bhansali *et al.* 2016, Heo & Lee 2020) until we reported that TM4SF3 associates with and mutually stabilizes AR in prostate cancer cells (Bhansali *et al.* 2016). More recently, we discovered that TM4SF3 interacts with and stabilizes not only AR but also AR-V7 (Khatiwada *et al.* 2023). Interestingly, we also determined that TM4SF3 interaction with AR or AR-V7 results in the regulation of gene expression through the recruitment of TM4SF3 to target promoters of AR or AR-V7 (Khatiwada *et al.* 2023). Furthermore, we verified that the TM4SF3 interaction with AR or AR-V7 is direct and were able to map the interaction domain of TM4SF3 and the common interaction domain of AR and AR-V7 (Khatiwada *et al.* 2023).

It is clear from our recent study that TM4SF3 pro-cancer functions depend on its ability to interact with AR or AR-V7 (Khatiwada *et al.* 2023), suggesting that targeting this interaction may be an effective novel therapy for prostate cancer. To do this, we considered a peptide-based approach to disrupt the TM4SF3 interaction with AR and AR-V7. Thus, we designed two peptides based on the interaction domain found within the TM4SF3 or AR protein and hypothesized that such peptides would bind to the target protein and prevent TM4SF3 interaction with either AR or AR-V7, thus destabilizing all three proteins and disrupting their pro-cancer functions. Indeed, both peptides disrupted TM4SF3 interaction with AR or AR-V7, resulting in the degradation of all proteins. The peptides also inhibited the corecruitment of TM4SF3 with AR or AR-V7 to target gene promoters, leading to attenuated gene expression in prostate cancer cells. Hence, treatment with either peptide reduced the proliferation and migration of and increased apoptosis of prostate cancer cells. Our study here identified a new approach to treating CRPC, by targeting the TM4SF3 interaction with AR or AR-V7.

Materials and methods

Cell culture and androgen treatment

LNCaP, PC-3, C81, CWR, HEK-293, and PrEC cells (From ATCC passage 9–35 for all cells) were purchased from ATCC and cultured as previously described (Khatiwada *et al.* 2020). R49F cells were kindly provided to us by Dr Amina Zubeidi (Vancouver Prostate Centre, BC, Canada) and were grown in RPMI-140 containing 10% fetal bovine serum (FBS), both from Thermofisher Scientific, penicillin–streptomycin, and 10 μM enzalutamide (Cayman Chemical). For androgen treatment, cells were grown for 48 h in RPMI-1640 or DMEM (HEK-293) containing 2% DCC and treated with either ethanol or 10 nM R1881 (Sigma-Aldrich).

Peptide treatment

All peptides used in this study were synthesized by BioBasic at ≥95% purity and dissolved in DMSO (Thermofisher Scientific). Peptide AT1 and TA1 were designed based on AR amino acids 141–162 and TM4SF3 amino acids 102–119, respectively, that are essential and sufficient for the AR/TM4SF3 interaction. Both peptides have eight arginine residues in the N-terminus that facilitate cell translocation (Dixon *et al.* 2016). Biotin-tagged peptides AT1 and TA1 were used for immunofluorescence and pulldown experiments.

Cell proliferation

For proliferation assay, LNCaP, stable LNCaP cells expressing AR, AR-V7 or TM4SF3, CWR-22RV1, R49F, PC-3, and PrEC cells were grown in 96-well plates (10000 cells/well) and treated with the indicated concentration of peptide TA1, peptide AT1, enzalutamide, or DMSO (vehicle) as indicated in the experiments. The MTT assay was used as before (Bhansali *et al.* 2016) to determine cell numbers on days 0, 2, and 4.

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

Apoptosis assay

The apoptosis assay was done in LNCaP and CWR cells following 20 μM treatment with peptide TA1 or AT1 for 4 days using annexin V-FITC/propidium iodide apoptosis kit (Invitrogen) or caspase 3/5 apoptosis kit (Invitrogen) following the manufacturer's protocol. Cell apoptosis was analyzed by LSRFortessa (BD Biosciences) using appropriate controls.

Soft agar assay

LNCaP and CWR-22Rv1 cells were plated at 5000 cells/well, after mixing 0.3% agar with RPMI medium supplemented with 10% FBS, on the surface of base agar (0.5% agar with RPMI and 10% FBS). RPMI medium was added twice a week along with the treatment of peptides (TA1 or AT1) or enzalutamide. After 28 days, colonies were counted using dissecting microscopes (LS stereo) from Olympus after being stained with crystal violet solution (0.05%) for 1 h followed by multiple washings with PBS.

Migration assay

LNCaP and CWR-22RV1 cells were treated with different concentrations of peptide for 48 h, after which cells were trypsinized and resuspended to 100,000 cells/mL. The resuspended cells were plated on the top chamber of the Cell CytoSelect 24-Well Cell Migration and Invasion Assay Combo kit, 8 μm (fluorometric quantitation) (Cell Biolabs) and, after 24 h, cell migration was measured following the manufacturer's protocol. Cell migration numbers were normalized to cell number as measured by MTT assay.

Western blotting

Western blotting was performed as described (22). Western blotting was performed using antibodies against AR, HA, or AR-V7 antibody, all from Cell Signaling Technology, against TM4SF3 or β-actin proteins, both Thermofisher Scientific, and against FLAG, from Sigma-Aldrich.

Peptide pulldown

Biotin-TA1 or biotin-AT1 was incubated overnight at 4°C with whole-cell extracts obtained from LNCaP or CWR-22Rv1 cells using M-Per™ (Thermofisher Scientific). Pierce™ NeutrAvidin™ Agarose beads (Thermofisher Scientific) were used to pulldown biotin-TA1 or biotin-AT1 and any bound proteins after a 2-h incubation. Bound proteins were eluted from the beads using Laemmli buffer and were subjected to western blotting for AR, TM4SF3, or AR-V7.

Immunoprecipitation

TA1 or AT1 was incubated overnight at 4°C with whole-cell extracts obtained from CWR-22Rv1 cells using M-Per™. Anti-AR antibody (Cell Signaling Technology) or IgG and Protein A/G agarose beads, both from Santa Cruz Biotechnology, were also added. Bound proteins were eluted from the beads using Laemmli buffer and were subjected to western blotting for AR, TM4SF3, or AR-V7.

BiFC and immunofluorescence

For the biomolecular fluorescence complementation assay (BiFC) assay, HEK-293 cells were cotransfected with different BiFC constructs as previously described (Xu *et al.* 2015, Khatiwada *et al.* 2023). Briefly, HEK-293 cells transfected with different BiFC constructs in the absence or presence of TA1 or AT1 for 48 h the cells and green fluorescence was observed under an Olympus microscope. Immunofluorescence was used to study the subcellular localization of the peptides in LNCaP and CWR-22Rv1 cells, which were treated with biotin-TA1 or biotin-AT1. Anti-biotin antibody (Santa Cruz Biotechnology) was used in LNCaP cells and CWR-22Rv1 cells to detect the peptides and anti-AR (Cell Signaling Technology), anti-AR-V7 (Cell Signaling Technology), and anti-TM4SF3 (Fisher Scientific) antibodies were also used in CWR-22Rv1 cells to measure colocalization. Secondary antibodies used were anti-rabbit secondary conjugated to Alexa Fluor 488 and anti-mouse secondary antibody conjugated to Alexa Fluor 546 (both from Life Technologies). Cells were also stained with DAPI for the detection of nuclei and observed on a Leica confocal microscope.

RNA isolation and qRT-PCR

Prostate cancer cells were grown overnight before treatment with TA1, AT1, or enzalutamide for 48 h. RNA isolation was performed using the TRIzol reagent (Invitrogen) following the manufacturer's instructions and real-time quantitative RT-PCR (qRT-PCR) was done using iQ SYBR Green Supermix (Bio-Rad,) and

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

the results were calculated 2. The PCR primers were purchased from IDT Technologies and the upstream and downstream primers, respectively, used for each gene were as follows: GAPDH, 5′-CGACCACTTTGTCAAGCTCA-3′, and 5′-AGGGGAGATTCAGTGTGGTG-3′; AR, 5′-GCATGGCAGAGTGCCCTATC-3′ and 5′-TCCCAGAGTCATCCCTGCTTCAT-3′); AR-V7, 5′-AAGAGCCGCTGAAGGGAAAC-3′ and 5′-TGCCAACCCGGAATTTTTCTC-3′; TM4SF3, 5′-GGCTTCCTGGGATGCTGCGG-3′ and 5′-GTCGCCACCTGCAGGAGCAG-3′; PSA, 5′-GCAGCATTGAACCAGAGGAG-3′ and 5′-CCCATGACGTGATACCTTGA-3′; TMPRSS2, 5′-CCTCTAACTGGTGTGATGGCGT-3′ and 5′-TGCCAGGACTTCCTCTGAGATG-3′; IGF1, 5′-CAACATCTCCCATCTCTCTG-3′ and 5′-GAAATCACAAAAGCAGCACT-3′; ADAM9A, 5′- GAATGCACAAGAACCACAAT-3′ and 5′-TAGGAAGCTACTAGGAGACA-3′; RAP2A, 5′-GATTCAGAGGCCTTCTAGTG-3 and E2F7, 5′-TGTATCTTTAAGGAAGCCCT-3′ and 5′-CGTCGACGTTCAACATTAAG-3′. qRT-PCR measurements were quantified following the $2^{-\Delta\Delta Ct}$ method (Livak method) and are given relative to GAPDH expression and are representing the average of three replicates plus standard deviations.

RNA sequencing

Prostate cancer cells were grown overnight before treatment with the vehicle (DMSO), 20 μM TA1, 20 μM AT1, or 10 μM enzalutamide for 48 h and RNA was isolated as described above. The RNA was sent to the UC Sequencing Core of the University of Cincinnati, which performed an RNA-Seq analysis using the NGS (Illumina Platform). Two replicates were used for each condition and BaseSpace (Illumina) Informatics analysis was performed only using a 2-fold change as the cutoff for changes in gene expression.

Chromatin immunoprecipitation

Prostate cancer cells were grown overnight before treatment with peptides or enzalutamide for 48 h. Chromatin immunoprecipitation assays were performed using SimpleChIP Enzymatic Chromatin IP Kit (Cell signaling Technology) following the manufacturer's protocol. The antibodies used in the IP experiments were against AR Carboxy Terminal (Cell Signaling Technology), AR-V7 (Cell Signaling Technology), TM4SF3 (Fisher Scientific), or IgG (Santa Cruz Biotechnology). After reverse cross-linking and DNA purification (zymo), immunoprecipitated DNA was quantified by qRT- PCR using using iQ SYBR Green Supermix (Bio-Rad) with primers for PSA, 5′-GACAACTTGCAAACCTGCTC-3′ and 5′-GATCCAGGCTTGCTTACTGT-3′; TMPRSS2, 5′-TGGTCCTGGATGATAAAAAAA GTTT-3′ and 5′-GACATACGCCCCACAACAGA-3′; IGF1, 5′-ACACACAAGCCAGCAGAGAA-3′ and 5′-CTGCAGTCAGCTGTGATCGT-3′; ADAM9A, 5′-TGTTTGTTTAGCTGTAACCATCAGA-3′ and 5′-TGTCCCTTTGGGAAAAACAC-3′; E2F7, 5′-TGGCATGTTAGGTGGATGGT-3′ and 5′-TTTTCCAGAGGCACCAAGCC-3′; RAP2A, 5′-TGGCTAAGCCCTCCATTACA-3′ and 5′-AGGGACGTGGCTACAGATTG-3′, and MMP1, 5′-TGGCCTTTGTCTTCTTTCTCA-3′ and 5′-GCAACACCAAGTGATTCCAA-3′.

Statistical analysis

All experiments were done at least three times and the data represented in the bar graphs and charts are averages plus standard deviation. The Student's *t*-test was performed to compare the difference between any pair of data and to calculate *P*-values.

Ethical statement

This study has been approved by the University of Toledo IBC (Institutional Biosafety Committee).

Results

Peptides were designed based on the interaction domains within AR and TM4SF3

We previously published that AR interacts with TM4SF3 in prostate cancer cells, resulting in mutual stabilization of both proteins (Bhansali *et al.* 2016). More recently, we reported that TM4SF3 also interacts with AR-V7 (Khatiwada *et al.* 2023), a splicing variant that lacks the ligand-binding domain (LBD) (Guo *et al.* 2009, Sun *et al.* 2010, Qu *et al.* 2015). In that previous study (Khatiwada *et al.* 2023), we also mapped the interaction regions of AR (amino acids 141-162) and TM4SF (amino acids 101-119) and learned that AR-V7 has the same region as AR and also interacts with TM4SF3. In this study, we learned that deletion of the interaction region from either

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

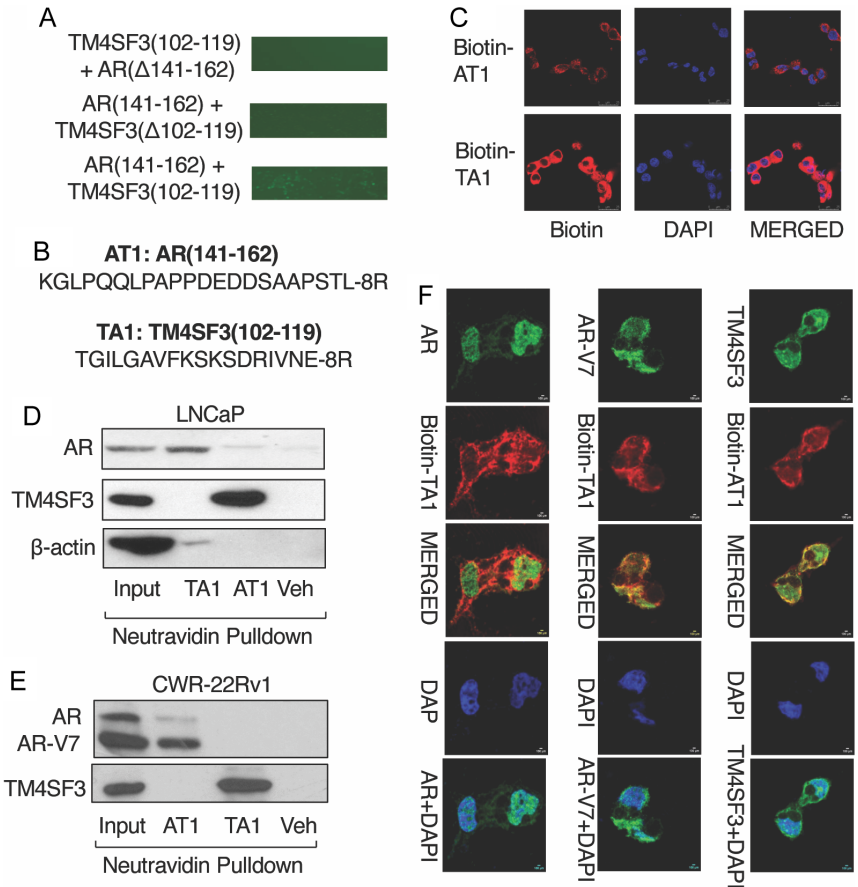

Figure 1
Peptides based on the interaction domains within AR and TM4SF3 associate with their target proteins. (A) HEK-293 cells were transfected with VN-AR and TM4SF3-VC truncations and deletions, as indicated, and were visualized for a BiFC interaction using an Olympus microscope. (B) Sequences for the AT1 and TA1 peptides are given. (C) LNCaP cells grown on full serum were treated with 20 μM biotin-AT1 or biotin-TA1 and were visualized for cell localization in a confocal microscope using appropriate primary and secondary antibodies. Whole-cell extracts from (D) LNCaP or (E) CWR-22Rv1 cells were incubated with the vehicle (Veh) or 50 μM biotin-TA1 or biotin-AT1 and subjected to neutravidin–agarose (NA) pulldown. Western blotting was used to detect AR, TM4SF3, and AR-V7; β-actin was used as a negative control. (F) CWR-22Rv1 cells were treated with 20 μM biotin-TA1 or biotin-AT1 and subjected to immunocytochemistry using an anti-AR, anti-AR-V7, anti-TM4SF3, or anti-biotin antibody to measure the subcellular colocalization of peptide TA1 with endogenous AR or AR-V7 or peptide AT1 with endogenous TM4SF3. DAPI staining (blue) was used stain the nuclei and merged images are shown of (C) biotin-TA1 or biotin-AT1 with DAPI or (F) biotin-TA1 with AR or AR-V7 or biotin-AT1 with TM4SF3, as indicated.

AR (Δ141-162) or TM4SF3 (Δ101-119) abolished their interaction (Fig. 1A), as measured by the BiFC assay that was used earlier (Khatiwada *et al.* 2023). These two interaction regions are not only required for the AR/TM4SF3 interaction, but importantly they are sufficient (Fig. 1A). Hence, we designed peptides TA1 (TM4SF3 amino acids 102–119) and AT1 (AR amino acids 120–140) (Fig. 1B), with the expectation that TA1 can physically bind to AR and AT1 to TM4SF3. Both peptides were synthesized with eight arginines in the C-terminus, which has been previously shown to act as a membrane-translocation signal (Tung & Weissleder 2003). As shown in Fig. 1C, peptides TA1 and AT1 can translocate into prostate cancer cells when added to the cell medium. To verify that these two peptides can interact with the target protein, we used TA1 and AT1 tagged with Biotin at the C-terminus in a streptavidin–agarose pulldown experiment. Indeed, biotin-TA1 was able to pulldown AR and biotin-AT1 can bring down TM4SF3 from an LNCaP nuclear extract (Fig. 1D). Importantly, TA1 also pulled down AR-V7, as well as AR, from CWR-22Rv1 nuclear extract (Fig. 1E). Immunocytochemistry experiments showed that TA1 co-localized with AR and AR-V7 and AT1 colocalized with TM4SF3 in CWR-22Rv1 cells (Fig. 1F). These data collectively show that TA1 associates with AR and AR-V7 and AT1 with TM4SF3 both in vitro and in cells.

Peptides TA1 and AT1 block the interaction of TM4SF3 with AR or AR-V7 and promote their degradation

To determine if the peptides can disrupt the AR/TM4SF3 interaction, we first used the BiFC assay. Both peptides TA1 and AT1 markedly blocked the AR/TM4SF3 or AR-V7/TM4SF3 interaction, as measured by BiFC assay (Fig. 2A). Importantly, these two peptides did not affect another BiFC interaction between the DNA repair proteins BARD1 and BRCA1 (Chen *et al.* 2018) (Supplementary Fig. 1A, see section on supplementary materials given at the end of this article), strongly suggesting that TA1 and AT1 specifically target the AR/TM4SF3 interaction. A negative control peptide had no effect on TMSF3 interaction with either AR or AR-V7 (Supplementary Fig. 1B), showing that the disrupting activity of TA1 or AT1 is not due to a nonspecific peptide effect. The peptides were also

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

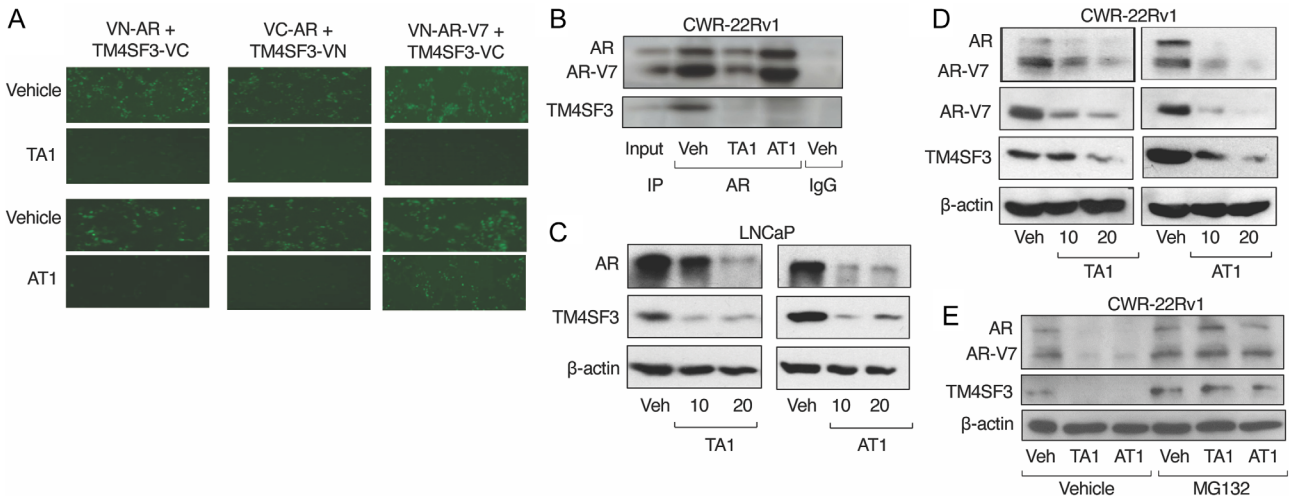

Figure 2

Peptides TA1 and AT1 block the interaction of TM4SF3 with AR or AR-V7 and promote the degradation of all three proteins. HEK-293 cells were transfected with (A) VN-AR, VN-AR-V7, and TM4SF3-VC, as indicated, before treatment with the vehicle (DMSO) or 10 μM peptide TA1 or AT1. After 24 h, the cells were visualized in an Olympus microscope for a BiFC interaction. (B) CWR-22Rv1 cells were treated with the Veh (vehicle) or 20 μM TA1 or AT1 and subjected to an immunoprecipitation using anti-AR antibody or IgG as a negative control. Western blotting was used to detect AR, AR-V7, and TM4SF3. (C) LNCaP or (D) CWR-22Rv1 cells were treated with Veh (the vehicle) or 10 or 20 μM TA1 or AT1 and subjected to a western blot to measure AR, AR-V7, TM4SF3, and β-actin. (E) CWR-22Rv1 cells were treated with the vehicle or 20 μM TA1 or AT1 and in the presence or absence of MG132 and subjected to subjected to a western blot to measure AR, AR-V7, TM4SF3, and β-actin, which was used as a loading control.

studied in a co-immunoprecipitation (co-IP) experiment, in which TA1 and AT1 equally inhibited the co-IP of TM4SF4 by IP of AR or AR-V7 (Fig. 2B); TA1 also reduced IP of AR and AR-V7 (Fig. 2B), possibly reflecting that peptide TA1 binding to AR aa 141-162 is disrupting binding of antibody, which binds to the AR N-terminus. Together, the BiFC and co-IP data clearly show that TA1 and AT1 can disrupt the physical interaction of TM4SF3 with either AR or AR-V7.

Since the AR/TM4SF3 interaction stabilizes both proteins (Khatiwada *et al.* 2023), disruption of this interaction is expected to lead to protein degradation. Indeed, treating cells with either TA1 or AT1 led to significantly reduced protein levels of AR and TM4SF3 in LNCaP cells (Fig. 2C) and TM4SF3, AR, and AR-V7 in CWR-22Rv1 cells (Fig. 2D). To verify that the peptides induced protein degradation, the experiment was repeated with the proteasomal inhibitor MG132, which was able to rescue the protein levels (Fig. 2E).

Peptides TA1 and AT1 disrupt the nuclear functions of AR and AR-V7 in prostate cancer cells

AR and AR-V7 affect the biology of prostate cancer cells by regulating the expression of genes (Lu *et al.* 2015). As expected, TA1 and AT1 inhibited the expression of AR target genes PSA, TMPRSS2, IGF1, and ADAM9A in LNCaP cells, with TA1 having a stronger effect than AT1 (Fig. 3A); the peptides had no significant effect on the gene expression of AR or TM4SF3 in LNCaP cells (Supplementary Fig. 2). Both peptides blocked the expression of AR and/or AR-V7 target genes also in CWR-22Rv1 cells (Fig. 3B), with TA1 again having a stronger effect. Next, we compared the effects of peptides to enzalutamide, the antiandrogen that is currently the most effective therapy for CRPC (Tran *et al.* 2009). In LNCaP cells, TA1 was as effective as enzalutamide at inhibiting gene expression induced by AR, with AT1 being generally weaker (Fig. 3C). In contrast, the expression of genes in enzalutamide-resistant CWR-22Rv1 cells targeted by either AR or AR-V7 (PSA and TMPRSS2) or AR-V7 alone (E2F7 and RAP2A) was largely unaffected by enzalutamide as expected, while TA1 and AT1 repressed these genes (Fig. 3D). Finally, TA1 and AT1 blocked the expression of four AR target genes in R49F cells, another enzalutamide-resistant cell line (Fig. 3E).

Since both enzalutamide and TA1 repress AR, we used RNA-Seq analysis to measure global gene expression to compare the negative effect of peptide to enzalutamide in hormone-dependent LNCaP cells and enzalutamide-resistant CWR-22Rv1 cells. Differential gene expression analysis indicated that the total number of repressed genes decreased by nearly 2-fold in CWR-22Rv1 cells (736 genes) as compared to LNCaP cells

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

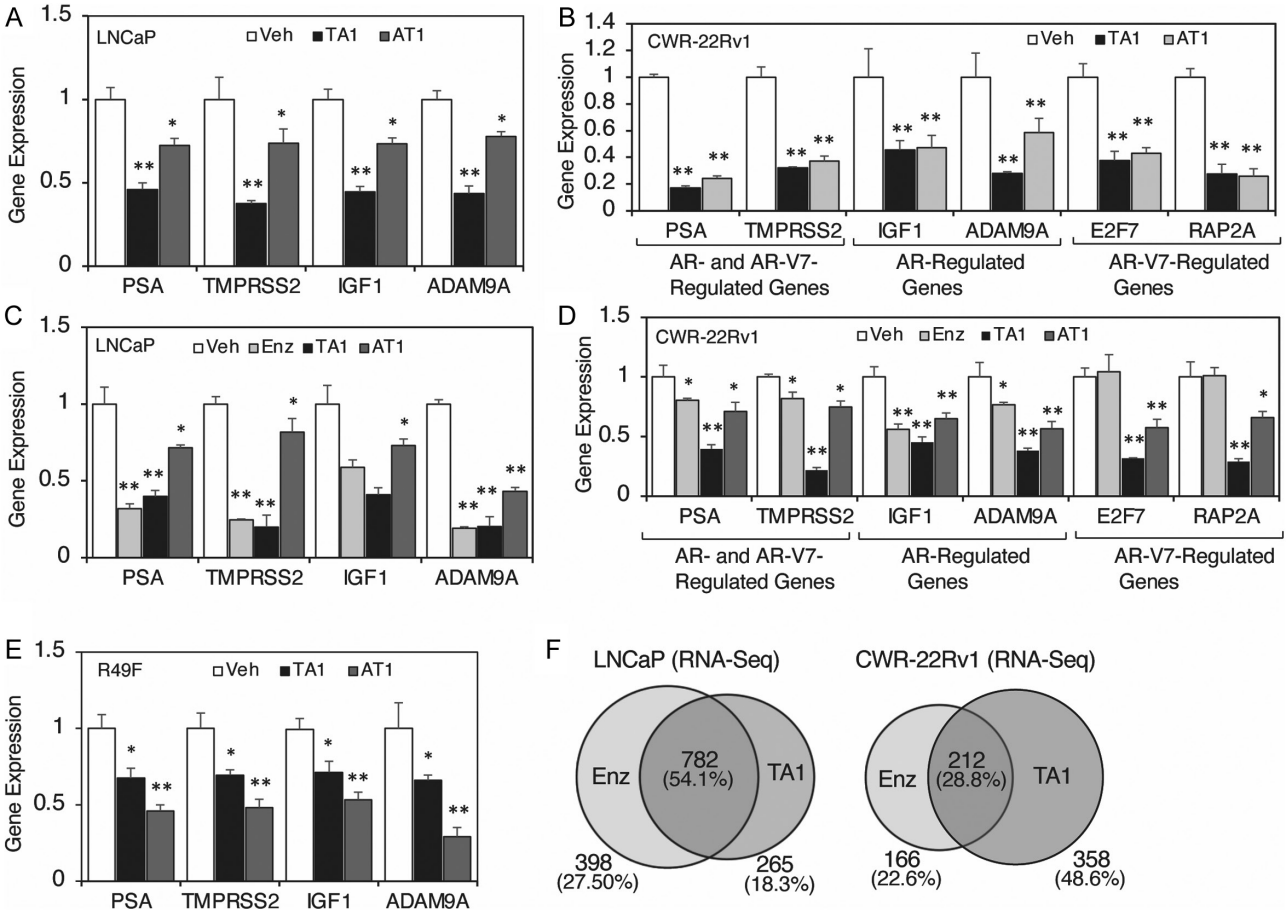

Figure 3

Peptides TA1 and AT1 reduce the expression of AR and AR-V7 target genes in prostate cancer cells. (A, C, F) LNCaP, (B, D, F) CWR-22Rv1, or (E) R49F were grown in 10% serum and treated with the vehicle (Veh), 20 μM TA1, 20 μM AT1, or 10 μM enzalutamide (Enz), as indicated, for 48 h and subjected to (A, B, C, D) qRT-PCR to measure the expression of AR and AR-V7 target genes or to (F) RNA-Seq analysis to measure global gene expression. Data points represent averages of three replicates plus standard deviations. Asterisks indicate statistical significance (*P < 0.05; **P< 0.01) of enzalutamide or peptides relative to the vehicle.

(1445 genes) (Fig. 3F). Enzalutamide-repressed genes was reduced in number by 67% when comparing LNCaP cells (1140 genes) to CWR-22Rv1 cells (378 genes) while TA1-repressed genes was reduced by only 46% (1047 to 570 genes), indicating that CWR-22Rv1 cells develop a greater resistance to enzalutamide than TA1. Further supporting this conclusion is our finding that 54.1% of genes were repressed by both enzalutamide and TA1 in LNCaP cells and only 28.8% in CWR-22Rv1 cells. Collectively, these data suggest that the differential sensitivity CWR-22Rv1 cells have to enzalutamide and TA1 is due to the differential inhibitory effects of these two molecules on gene expression. As suggested by the data of Fig. 3F, there are many genes in CWR-22Rv1 cells repressed by TA1 that are not affected by enzalutamide.

AR regulation of gene expression requires its recruitment to the promoters of target genes. Using chromatin immunoprecipitation (ChIP), we discovered that peptide TA1 strongly interfered with the recruitment of AR and TM4SF3 to AR target genes in LNCaP cells (Fig. 4A). TA1 and AT1 were about as effective as enzalutamide in blocking binding of AR and TM4SF3 to AR target genes in LNCaP cells (Fig. 4B). In CWR-22Rv1 cells, TA1 and AT1 strongly inhibited recruitment of AR-V7 and TM4SF3 to the AR-V7 target genes E2F7 and RAP2A (Fig. 4C); enzalutamide had no effect on AR-V7 recruitment, as expected, but interestingly, it had a small but significant negative effect on TM4SF3 recruitment (Fig. 4C). In contrast, enzalutamide was equally active as the two peptides in blocking the recruitment of AR, TM4SF3, and, intriguingly, AR-V7 to PSA and TMPRSS2, which are target genes of both AR and AR-V7 (Fig. 4C). Taken together, these data clearly indicate that peptides TA1 and AT1 interfere with the nuclear functions of

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

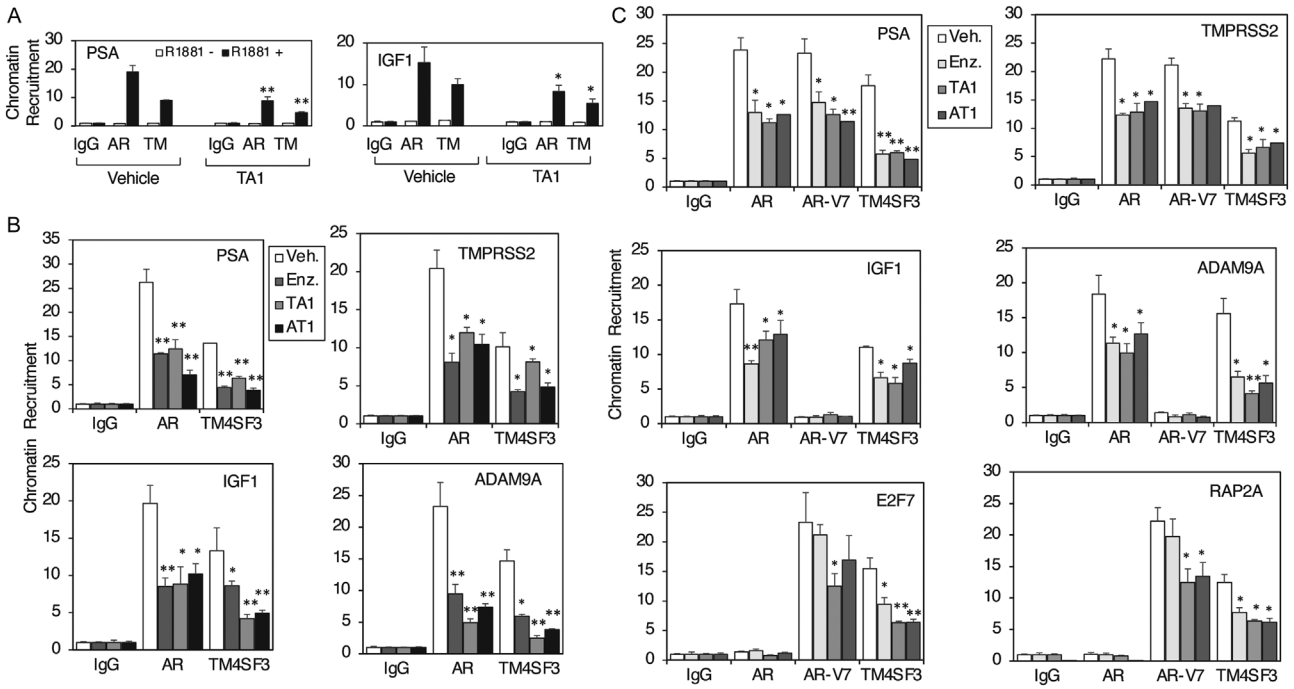

Figure 4

Peptides TA1 and AT1 reduce the recruitment of AR, AR-V7, and TM4SF3 to the promoters of target genes. LNCaP cells grown in (A) 2% serum in the presence of ethanol (-, vehicle) or 10 nM R1881 or in (B) 10% serum or (C) CWR-22Rv1 cells grown in 10% serum were treated with the vehicle (Veh), 20 μM TA1, 20 μM AT1, or 10 μM enzalutamide (Enz), as indicated, for 48 h before chromatin extraction, and subjected to ChIP assays using antibodies against AR, AR-V7, TM4SF3, or IgG (negative) on the AR target genes IGF1 and ADAM9, the AR-V7 target genes E2F7 and RAP2A, and the target genes of both proteins PSA and TMPRSS2. ChIP values were calculated as fold enrichment relative to 10% input and were normalized with IgG control. Each value shown in the bar graphs is the average of three replicates plus standard deviations. Asterisks indicate statistical significance (*P < 0.05, **P < 0.01) of the recruitment of AR, AR-V7, or TM4SF3, peptide TA1 or AT1 or enzalutamide relative to recruitment with vehicle alone.

both AR and AR-V7 in both enzalutamide-sensitive and most importantly, enzalutamide-resistant prostate cancer cells.

Peptides TA1 and AT1 are cytotoxic to prostate cancer cells

AR and AR-V7 are essential for the survival and growth of prostate cancer cells (Simental *et al.* 1991, Gelmann 2002, Huang & Tindall 2002, Lonergan & Tindall 2011). Since peptides TA1 and AT1 downregulate the levels of both AR proteins and disrupt their nuclear functions, it is expected that these peptides would disrupt the viability of prostate cancer cells. This was first examined first in LNCaP cells, which were strongly inhibited in their growth in a dose-dependent manner by both peptides TA1 (Fig. 5A) and AT1 (Fig. 5B). TA1 (Fig. 5A) and AT1 (Fig. 5B) had similar cytotoxic activities on hormone-independent C81 cells. Both LNCaP and C81 cells are similarly sensitive to enzalutamide and the peptides (Supplementary Fig. 3A and B). Importantly, CWR-22Rv1 (Supplementary Fig. 3A and B) and R49F cells, which are resistant to enzalutamide, were highly sensitive to peptides TA1 and AT1 (Figs. 5A, B, Supplementary Fig. 3A and B). On the other hand, TA1 and AT1 had little, if any, effect on the growth of AR-negative PC-3 (Supplementary Fig. 4A), PrEC (primary prostate epithelial) (Supplementary Fig. 4B), and HEK-293 (Supplementary Fig. 4C) cells. Collectively, these data suggest that the TA1 and AT1 are cytotoxic to prostate cancer cells because of the negative effect on AR protein levels. It is also possible that the peptides are killing cells through an off-target effect, which we examined using a rescue experiment with overexpressed exogenous proteins. Importantly, overexpression of either AR or AR-V7 resulted in full rescue of LNCaP cells treated with either peptide TA1 or AT1 (Fig. 5C), while vehicle-treated cells exhibited a much smaller effect (Fig. 5C). These data strongly support the notion that the peptides are killing cells via reduced levels of AR and argue against off-target effects. Overexpression of AR or AR-V7 also rescued enzalutamide-treated cells (Fig. 5C) as expected. Since the peptides are blocking the growth of prostate cancer cells, it is possible that they induce apoptosis. Indeed, TA1 or AT1

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

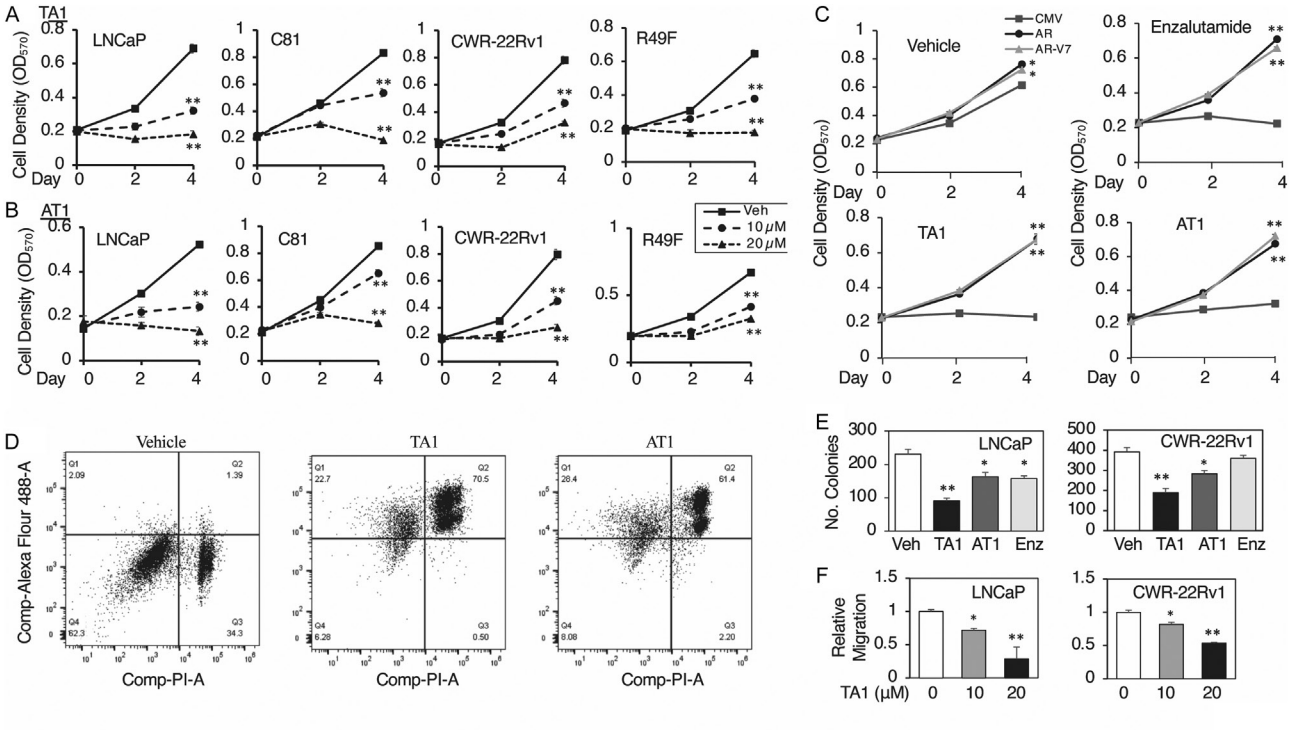

Figure 5

Peptides TA1 and AT1 reduce the viability of prostate cancer cells. LNCaP, C81, CWR-22Rv1, and R49F cells were treated with 0 (vehicle), 10 or 20 μM TA1 (A) or AT1 (B). (C) LNCaP cells were infected with lentivirus expressing empty (CMV), AR, or AR-V7 and treated with the vehicle, 10 μM enzalutamide, 20 μM TA1, or 20 μM AT1, as indicated. Cell density was measured on days 0, 2, and 4 using the MTT assay. (D) LNCaP cells were treated with the vehicle or 20 μM TA1 or AT1 were analyzed for apoptosis by measuring annexin V using flow cytometry. The cells in Q1 represent necrotic, Q2 apoptotic, Q3 early apoptotic, and Q4 viable cells. LNCaP and CWR-22Rv1 cells were treated with the vehicle (Veh), 20 μM TA1 or AT1, or 10 μM enzalutamide (Enz) and measured for (E) growth on soft agar or (F) migration. The migrated cells were normalized for proliferation and are shown as relative to proliferation. Each value shown is the average of three replicates plus standard deviations. Asterisks indicate statistical significance (*$P < 0.05$, **$P < 0.01$) of the recruitment of (A) peptide-treated cells as compared to the vehicle, (C) cells transfected with AR or AR-V7 as compared to empty (E) cells treated with peptide or enzalutamide as compared to the vehicle, or (F) cells treated with different concentrations of peptide as compared to the vehicle.

treatment induced the apoptosis of LNCaP cells, as monitored by measuring the levels of annexin V (Fig. 5D) or caspase 3/7 (Supplementary Fig. 5) activity using fluorescence-activated cell sorting analysis.

AR activity in prostate cancer cells supports not only cell survival and proliferation but also transformation (Zhu *et al.* 2011) and migration (Deng *et al.* 2017). As shown in Fig. 5E, both peptide TA1 and AT1 markedly repressed the malignant transformation of LNCaP and CWR-22Rv1 cells, as measured by soft agar assay, with TA1 being stronger than either AT1 or enzalutamide. TA1 also inhibited in a dose-dependent manner the migration of both LNCaP and CWR-22Rv1 cells (Fig. 5F).

Discussion

Protein–protein interactions (PPIs) are key to the vital functions that proteins serve in cells (Athanasios *et al.* 2017) and involved in all aspects of protein functions, including their important roles in diseased states like cancer (Athanasios *et al.* 2017). Interestingly, about 650,000 PPIs have been estimated to exist within the human interactome that are involved in disease (Ottmann 2016). AR is the major regulator of the initiation and progression of prostate cancer (Simental *et al.* 1991, Gelmann 2002, Huang & Tindall 2002, Lonergan & Tindall 2011) and carries out its pro-cancer functions by interacting with many diverse proteins (Culig & Santer 2018). Despite this, there is only one reported example of a molecule targeting a PPI involving AR, the natural product ASC-J9 that disrupts AR-ARA55 interaction (Lai *et al.* 2013).

We explored the possibility of targeting an important PPI that AR has with a novel protein, TM4SF3, a transmembrane protein (Huang *et al.* 2005) that is also found in soluble form in prostate cancer cells which interacts with AR (Bhansali *et al.* 2016). Our previous

data show that cytosolic TM4SF3 interacts directly and specifically with AR, where it is co-recruited to AR target genes and promotes AR activity on those genes (Khatiwada *et al.* 2023). Mapping studies identified the interaction domains to be found in the most variable regions of AR (the N-terminal domain) and TM4SF3 (Khatiwada *et al.* 2023), making us confident that we could target these two novel PPI regions. Since PPI generally cover large surfaces, the development of small molecules targeting PPIs continues to face challenges ((Hashimoto & Eichler 2015, Mabonga & Kappo 2020). In contrast to small molecules, peptides can bind to PPI with selectivity and specificity (Hashimoto & Eichler 2015, Mabonga & Kappo 2020). Thus, we developed peptides TA1 and AT1 that mimicked the newly identified PPI region of TM4SF3 or AR, respectively, and showed that they disrupt the AR/TM4SF3 interaction. Importantly, the amino acid sequences of peptides AT1 and TA1 are only found in their entirety in the proteins AR and TM4SF3, respectively, from which they were designed, reducing the possibility of off-target effects.

Since the AR/TM4SF3 interaction results in mutual stabilization of both proteins (Bhansali *et al.* 2016, Khatiwada *et al.* 2023), peptides disrupting this interaction were expected to cause proteasomal degradation of both proteins, which is what we observed with peptides TA1 and AT1. Hence, our peptides disrupt AR activity in prostate cancer cells by promoting its degradation, which is different from the mode of action of existing antiandrogens like enzalutamide and abiraterone (van der Kwast *et al.* 1991, Wilt *et al.* 2008). In addition, our peptides target a novel PPI, which is different from what ASC-J9 targets (Lai *et al.* 2013), and thus the peptides represent a new mechanism by which to downregulate AR in prostate cancer cells.

The new mechanism is also significant because it has relevance to the regulation of AR-V7, an AR variant that lacks the LBD and thus is resistant to both enzalutamide and abiraterone (Haile & Sadar 2011, Wadosky & Koochekpour 2017). In fact, AR-V7 expression may be responsible for nearly 20% of deadly CRPC (Haile & Sadar 2011, Wadosky & Koochekpour 2017). Since the PPI region of AR, that the peptide AT1 is based on, is also found on AR-V7, it was not surprising for us to discover earlier (Khatiwada *et al.* 2023) that TM4SF3 exhibits a similar interaction with AR-V7 and mutual stabilization that it has with AR. Hence, peptides TA1 and AT1 were also able to disrupt the AR-V7/TM4SF3 interaction and cause proteasomal degradation of AR-V7.

Our previous study (Khatiwada *et al.* 2023) showed that TM4SF3 regulates AR and AR-V7 by two distinct mechanisms following the physical interaction. First, TM4SF3 associates with and blocks the proteasomal degradation of AR and AR-V7. Second, the AR/TM4SF3 or AR-V7/TM4SF4 complex translocates into the nucleus, where TM4SF3 is corecruited to AR or AR-V7 target genes (Khatiwada *et al.* 2023). Peptides TA1 and AT1 inhibited the nuclear functions of TM4SF3, as measured by the recruitment of TM4SF3 and the AR proteins to their target genes and expression of AR or AR-V7 target genes. We used RNA-Seq here to measure the effect of peptides on global gene expression in prostate cancer cells. We identified several known AR target genes and many more unknown genes which were inhibited by enzalutamide in LNCaP cells but not in CWR-22Rv1 cells, providing a molecular basis for the enzalutamide resistance these cells display. Interestingly, these genes retained sensitivity to TA1. These findings with the peptides were expected since they block the TM4SF3 interaction with AR or AR-V7, which is necessary for both protecting the proteins from proteasomal degradation and regulation of gene expression. It is interesting that TA1 was generally stronger than AT1 in inhibiting the expression of several known AR target genes, while the two peptides were similarly cytotoxic to prostate cancer cells. These findings suggest that cell growth is not dependent on these known genes that are differentially affected by the two peptides. Instead, other unknown AR target genes may be important for cell growth, and these genes may be similarly inhibited by TA1 and AT1, something that can be studied in the future.

Disruption of AR functions is expected to lead to cytotoxicity of prostate cancer cells, as we found when we treated with TA1 or AT1 different stages of prostate cancer cells. Importantly, the peptides were cytotoxic to enzalutamide-resistant CWR-22Rv1 and R49F cells. Enzalutamide resistance comes from multiple mechanisms (Guo *et al.* 2009, Sun *et al.* 2010, Qu *et al.* 2015) and may include the expression of AR-V7 (Sramkoski *et al.* 1999) and hyperactivation of AR (Yamamoto *et al.* 2014). Since these two mechanisms may be responsible for resistance found in CRPC that typically leads to patient death (Sramkoski *et al.* 1999) and hyperactivation of AR (Yamamoto *et al.* 2014), our data here suggest that the new therapy based on our peptides can be effective against deadly CRPC. This argument is strengthened by the knowledge that the N-terminal PIP region is also found in the other twenty known AR variants, in addition to AR-V7, thereby making it possible that

https://eo.bioscientifica.com
https://doi.org/10.1530/EO-23-0010

TM4SF3 interacts with those other AR variants as it does with AR-V7. If this is the case, then the peptides can also kill prostate cancer cells that are enzalutamide-resistant due to the expression of the other AR variants. Future work can address possible interactions of TM4SF3 with other AR variants and the possible cytotoxic activity of peptides TA1 and AT1 against prostate cancer cells expressing various AR variants.

Although our data here show that peptides TA1 and AT1 have favorable activities against prostate cancer cells, particularly those resistant to enzalutamide, peptides in general do not make good drugs, with numerous well-known obstacles related to absorption, distribution, metabolism, and excretion (ADME) (Qvit *et al.* 2017, Drucker 2020). Thus, an additional important consideration in the future is to convert these peptide inhibitors into more drug-like compounds. This could include incorporation of unnatural amino acids, cyclization of peptides to restrain conformations, or identification of small molecule mimics of the peptides (Qvit *et al.* 2017, Drucker 2020).

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EO-23-0010.

Declaration of interest

There is no conflict of interest for all the authors.

Funding

This work was supported by a grant from the Department of Defense (W81XWH-17-1-0263).

Author contribution statement

PK performed experiments and analyzed data; UR performed experiments and analyzed data; MM performed experiments; ZH performed experiments; and LS conceived the study and wrote the paper.

References

Anami K, Oue N, Noguchi T, Sakamoto N, Sentani K, Hayashi T, Naito Y, Oo HZ & Yasui W 2016 TSPAN8, identified by Escherichia coli ampicillin secretion trap, is associated with cell growth and invasion in gastric cancer. *Gastric Cancer* **19** 370–380. (https://doi.org/10.1007/s10120-015-0478-z)

Athanasios A, Charalampos V, Vasileios T & Ashraf GM 2017 Protein-protein interaction (PPI) network: recent advances in drug discovery. *Current Drug Metabolism* **18** 5–10. (https://doi.org/10.2174/1389200218011701192048 32)

Bhansali M, Zhou J & Shemshedini L 2016 TM4SF3 and AR: A nuclear complex that stabilizes both proteins. *Molecular Endocrinology (Baltimore, Md.)* **30** 13–25. (https://doi.org/10.1210/me.2015-1075)

Chen CC, Feng W, Lim PX, Kass EM & Jasin M 2018 Homology-directed repair and the role of BRCA1, BRCA2, and related proteins in genome integrity and cancer. *Annual Review of Cancer Biology* **2** 313–336. (https://doi.org/10.1146/annurev-cancerbio-030617-050502)

Culig Z & Santer FR 2018 Studies on steroid receptor coactivators in prostate cancer. *Prostate Cancer. Methods and Protocols* 259–262.

Deng YL, Guo K, Zeng YK, Wu KH, Tang C & Zheng SB 2017 Effect of androgen receptor on IgG expression, proliferation and migration of prostate cancer cells in vitro. *Nan Fang Yi Ke Da Xue Xue Bao= Journal of Southern Medical University* **37** 388–392. (https://doi.org/10.3969/j.issn.1673-4254.2017.03.19)

Denmeade SR & Isaacs JT 2002 A history of prostate cancer treatment. *Nature Reviews. Cancer* **2** 389–396. (https://doi.org/10.1038/nrc801)

Dixon JE, Osman G, Morris GE, Markides H, Rotherham M, Bayoussef Z, El Haj AJ, Denning C & Shakesheff KM 2016 Highly efficient delivery of functional cargoes by the synergistic effect of GAG binding motifs and cell-penetrating peptides. *Proceedings of the National Academy of Sciences of the United States of America* **113** E291–E299. (https://doi.org/10.1073/pnas.1518634113)

Dong Z, Zhao L, Lu S, Xiong J & Geng Z 2016 Overexpression of TSPAN8 promotes tumor cell viability and proliferation in nonsmall cell lung cancer. *Cancer Biotherapy and Radiopharmaceuticals* **31** 353–359. (https://doi.org/10.1089/cbr.2016.2108)

Drucker DJ 2020 Advances in oral peptide therapeutics. *Nature Reviews. Drug Discovery* **19** 277–289. (https://doi.org/10.1038/s41573-019-0053-0)

Gelmann EP 2002 Molecular biology of the androgen receptor. *Journal of Clinical Oncology* **20** 3001–3015. (https://doi.org/10.1200/JCO.2002.10.018)

Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, Chen H, Kong X, Melamed J, Tepper CG, *et al.* 2009 A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion–resistant growth. *Cancer Research* **69** 2305–2313. (https://doi.org/10.1158/0008-5472.CAN-08-3795)

Haile S & Sadar MD 2011 Androgen receptor and its splice variants in prostate cancer. *Cellular and Molecular Life Sciences* **68** 3971–3981. (https://doi.org/10.1007/s00018-011-0766-7)

Hashimoto C & Eichler J 2015 Turning peptide ligands into small-molecule inhibitors of protein–protein interactions. *Chembiochem* **16** 1855–1856. (https://doi.org/10.1002/cbic.201500298)

Hemler ME 2014 Tetraspanin proteins promote multiple cancer stages. *Nature Reviews. Cancer* **14** 49–60. (https://doi.org/10.1038/nrc3640)

Heo K & Lee S 2020 TSPAN8 as a novel emerging therapeutic target in cancer for monoclonal antibody therapy. *Biomolecules* **10** 388. (https://doi.org/10.3390/biom10030388)

Huang H & Tindall DJ 2002 The role of the androgen receptor in prostate cancer. *Critical Reviewss in Eukaryotic Gene Expression* **12** 193–207. (https://doi.org/10.1615/critreveukaryotgeneexpr.v12.i3.30)

Huang S, Yuan S, Dong M, Su J, Yu C, Shen Y, Xie X, Yu Y, Yu X, Chen S, *et al.* 2005 The phylogenetic analysis of tetraspanins projects the evolution of cell-cell interactions from unicellular to multicellular organisms. *Genomics* **86** 674–684. (https://doi.org/10.1016/j.ygeno.2005.08.004)

Huggins C & Hodges CV 1972 Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA: a Cancer Journal for Clinicians* **22** 232–240. (https://doi.org/10.3322/canjclin.22.4.232)

Huggins C, Stevens R & Hodges CV 1941 Studies on prostatic cancer: II. The effects of castration on advanced carcinoma of the prostate gland. *Archives of Surgery* **43** 209–223. (https://doi.org/10.1001/archsurg.1941.01210140043004)

Khatiwada P, Kannan A, Malla M, Dreier M & Shemshedini L 2020 Androgen up-regulation of Twist1 gene expression is mediated by ETV1. *PeerJ* **8** e8921. (https://doi.org/10.7717/peerj.8921)

Khatiwada P, Rimal U, Han Z, Malla M, Zhou J & Shemshedini L 2023 The transmembrane protein TM4SF3 interacts with AR and AR-V7 and is recruited to AR target genes. *Endocrinology* **164**. (https://doi.org/10.1210/endocr/bqad048)

Lai KP, Huang CK, Chang YJ, Chung CY, Yamashita S, Li L, Lee SO, Yeh S & Chang C 2013 New therapeutic approach to suppress castration-resistant prostate cancer using ASC-J9 via targeting androgen receptor in selective prostate cells. *American Journal of Pathology* **182** 460–473. (https://doi.org/10.1016/j.ajpath.2012.10.029)

Lonergan PE & Tindall DJ 2011 Androgen receptor signaling in prostate cancer development and progression. *Journal of Carcinogenesis* **10** 20. (https://doi.org/10.4103/1477-3163.83937)

Lu J, Lonergan PE, Nacusi LP, Wang L, Schmidt LJ, Sun Z, van der Steen T, Boorjian SA, Kosari F, Vasmatzis G, *et al.* 2015 The cistrome and gene signature of androgen receptor splice variants in castration resistant prostate cancer cells. *Journal of Urology* **193** 690–698. (https://doi.org/10.1016/j.juro.2014.08.043)

Mabonga L & Kappo AP 2020 Peptidomimetics: a synthetic tool for inhibiting protein–protein interactions in cancer. *International Journal of Peptide Research and Therapeutics* **26** 225–241. (https://doi.org/10.1007/s10989-019-09831-5)

Ottmann C 2016 New compound classes: protein–protein interactions. *New Approaches to Drug Discovery* 125–138. (https://doi.org/10.1007/164_2015_30)

Qu Y, Dai B, Ye D, Kong Y, Chang K, Jia Z, Yang X, Zhang H, Zhu Y & Shi G 2015 Constitutively active AR-V7 plays an essential role in the development and progression of castration-resistant prostate cancer. *Scientific Reports* **5** 1–6. (https://doi.org/10.1038/srep07654)

Qvit N, Rubin SJS, Urban TJ, Mochly-Rosen D & Gross ER 2017 Peptidomimetic therapeutics: scientific approaches and opportunities. *Drug Discovery Today* **22** 454–462. (https://doi.org/10.1016/j.drudis.2016.11.003)

Siegel RL, Miller KD & Jemal A 2020 Cancer statistics, 2020. *CA: a Cancer Journal for Clinicians* **70** 7–30. (https://doi.org/10.3322/caac.21590)

Simental JA, Sar M, Lane MV, French FS & Wilson EM 1991 Transcriptional activation and nuclear targeting signals of the human androgen receptor. *Journal of Biological Chemistry* **266** 510–518. (https://doi.org/10.1016/S0021-9258(18)52466-2)

Sramkoski RM, Pretlow TG, Giaconia JM, Pretlow TP, Schwartz S, Sy MS, Marengo SR, Rhim JS, Zhang D & Jacobberger JW 1999 A new human prostate carcinoma cell line, 22R v1. *In Vitro Cellular and Developmental Biology. Animal* **35** 403–409. (https://doi.org/10.1007/s11626-999-0115-4)

Sun S, Sprenger CC, Vessella RL, Haugk K, Soriano K, Mostaghel EA, Page ST, Coleman IM, Nguyen HM, Sun H, *et al.* 2010 Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *Journal of Clinical Investigation* **120** 2715–2730. (https://doi.org/10.1172/JCI41824)

Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, *et al.* 2009 Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* **324** 787–790. (https://doi.org/10.1126/science.1168175)

Tung CH & Weissleder R 2003 Arginine containing peptides as delivery vectors. *Advanced Drug Delivery Reviews* **55** 281–294. (https://doi.org/10.1016/s0169-409x(02)00183-7)

van der Kwast TH, Schalken J, de Winter JAR, van Vroonhoven CC, Mulder E, Boersma W & Trapman J 1991 Androgen receptors in endocrine-therapy-resistant human prostate cancer. *International Journal of Cancer* **48** 189–193. (https://doi.org/10.1002/ijc.2910480206)

Wadosky KM & Koochekpour S 2017 Androgen receptor splice variants and prostate cancer: from bench to bedside. *Oncotarget* **8** 18550–18576. (https://doi.org/10.18632/oncotarget.14537)

Wang H, Rana S, Giese N, Büchler MW & Zöller M 2013 Tspan8, CD44v6 and alpha6beta4 are biomarkers of migrating pancreatic cancer-initiating cells. *International Journal of Cancer* **133** 416–426. (https://doi.org/10.1002/ijc.28044)

Wasson JH, Cushman CC, Bruskewitz RC, Littenberg B, Mulley AG & Wennberg JE 1993 A structured literature review of treatment for localized prostate cancer. Prostate Disease Patient Outcome Research Team. *Archives of Family Medicine* **2** 487–493. (https://doi.org/10.1001/archfami.2.5.487)

Wei L, Li Y & Suo Z 2015 TSPAN8 promotes gastric cancer growth and metastasis via ERK MAPK pathway. *International Journal of Clinical and Experimental Medicine* **8** 8599–8607. (https://doi.org/10.1038/s41467-019-10739-3)

Wilt TJ, Macdonald R, Rutks I, Shamliyan TA, Taylor BC & Kane RL 2008 Systematic review: comparative effectiveness and harms of treatments for clinically localized prostate cancer. *Annals of Internal Medicine* **148** 435–448. (https://doi.org/10.7326/0003-4819-148-6-200803180-00209)

Xu D, Zhan Y, Qi Y, Cao B, Bai S, Xu W, Gambhir SS, Lee P, Sartor O, Flemington EK, *et al.* 2015 Androgen receptor splice variants dimerize to transactivate target genes. *Cancer Research* **75** 3663–3671. (https://doi.org/10.1158/0008-5472.CAN-15-0381)

Yamamoto Y, Loriot Y, Beraldi E, Zhou T, Kim Y, Monia BP, Macleod AR, Fazli L, Zoubeidi A & Gleave ME 2014 Biologic consequences of androgen receptor (AR) activity in MDV3100-Resistant LNCaP cells and dependence on AR full length. *American Society of Clinical Oncology* **32** 74. (https://doi.org/10.1200/jco.2014.32.4_suppl.74)

Zhang HS, Liu HY, Zhou Z, Sun HL & Liu MY 2020 TSPAN8 promotes colorectal cancer cell growth and migration in LSD1-dependent manner. *Life Sciences* **241** 117114. (https://doi.org/10.1016/j.lfs.2019.117114)

Zhou Z, Ran YL, Hu H, Pan J, Li ZF, Chen LZ, Sun LC, Peng L, Zhao XL, Yu L, *et al.* 2008 TM4SF3 promotes esophageal carcinoma metastasis via upregulating ADAM12m expression. *Clinical and Experimental Metastasis* **25** 537–548. (https://doi.org/10.1007/s10585-008-9168-0)

Zhu C, Luong R, Zhuo M, Johnson DT, Mckenney JK, Cunha GR & Sun Z 2011 Conditional expression of the androgen receptor induces oncogenic transformation of the mouse prostate. *Journal of Biological Chemistry* **286** 33478–33488. (https://doi.org/10.1074/jbc.M111.269894)

Zhu R, Gires O, Zhu L, Liu J, Li J, Yang H, Ju G, Huang J, Ge W & Chen Y 2019 TSPAN8 promotes cancer cell stemness via activation of sonic Hedgehog signaling. *Nature Communications* **10** 1–14.

Received 3 May 2023
Accepted 29 August 2023
Available online 30 August 2023
Version of Record published 27 September 2023