Triptolide Inhibits the AR Signaling Pathway to Suppress the Proliferation of Enzalutamide Resistant Prostate Cancer Cells

Supplementary Materials and Methods

Plasmids

pCMV-Gal4DBD and p5xUAS-TATA-luciferase were gifts from Dr. J. Wong. pCMV–Gal4DBD-AR (1-558) was generated by subcloning appropriate PCR products from pcDNA3-AR into pCMV-Gal4DBD vector. pcDNA3-AR-FL and pcDNA3-AR-V7 were gifts from Dr H. Huang. pLVX-AR-V7 was generated by subcloning human AR-V7 into pLVX-puro vector. pLKO.1-shXPB-1 and pLKO.1-shXPB-2 were generated by subcloning shXPB into pLKO.1-puro vector. The sequences of shXPB and siXPB are listed in Supplementary Table S1.

MTT assay

MTT assay followed a previous report.[1] 5 x 10^3 cells/well were cultured in 96-well plate overnight, and then treated with individual or combined TPL and enzalutamide at various concentrations as indicated, using DMSO as the vehicle control and culture medium as the blank control. After the appropriate treatment time, 10 μl MTT solution per well was added in plate and incubated for 4 h at 37 ℃. Absorbance at 490 nm was examined using a microplate reader (BioTek Instruments). Each treatment was performed in triplicate and experiments were repeated at least 3 times.

SRB assay

SRB assay followed a previous report.[2] 22Rv1 or C4-2R cells were cultured in 96-well plates at a density of 3 × 10^3 or 4.5 × 10^3 cells/well respectively overnight. Cells were treated with individual or combined TPL and enzalutamide at various concentrations as indicated. After 48 h, cells were fixed with 100 μL of 10% trichloroacetic acid for 2 h and stained with 4 mg/mL sulforhodamine B (SRB, Sigma) in 1% acetic acid. The SRB in cells was dissolved in 10 mM

Tris-HCl and was measured at 560 nm. The half maximal inhibitory concentration (IC50) values were calculated using GraphPad Prism 6.0.

RNA isolation, reverse transcription, and quantitative real-time PCR

PCa cells were treated with TPL or enzalutamide as indicated. Total RNAs were extracted using TRIzol reagent (Invitrogen). 1 μg of RNA was converted to cDNA using a 1st Strand cDNA Synthesis kit (TaKaRa). Real-time PCR was performed using a SYBR Premix Ex TaqTM II kit (TaKaRa) and measured by ABI Stepone plus. The mRNA level of each gene was normalized to β-actin with the ΔΔCT method. The primer sequences for qRT-PCR are listed in Supplementary Table S1.

FACS analysis

LNCaP, C4-2/Luci and C4-2/AR-V7 cells grown in 6-well plates were treated with various doses of TPL for 24 h, and stained with Annexin V (AV) conjugated with FITC and propidium iodide (PI) using the Annexin V-FITC Apoptosis Assay Kit (C1062, Beyotime) following the manufacturer's instructions. Stained cells were analyzed with a Cyflow Cube flow cytometer (PARTEC). Data were analyzed using FlowJO 7.6.5 software.

Measurement of PSA

PSA levels were measured in the supernatants using a PSA ELISA Kit (CSB-E04768h, Cusabio) following the manufacturer's instructions.

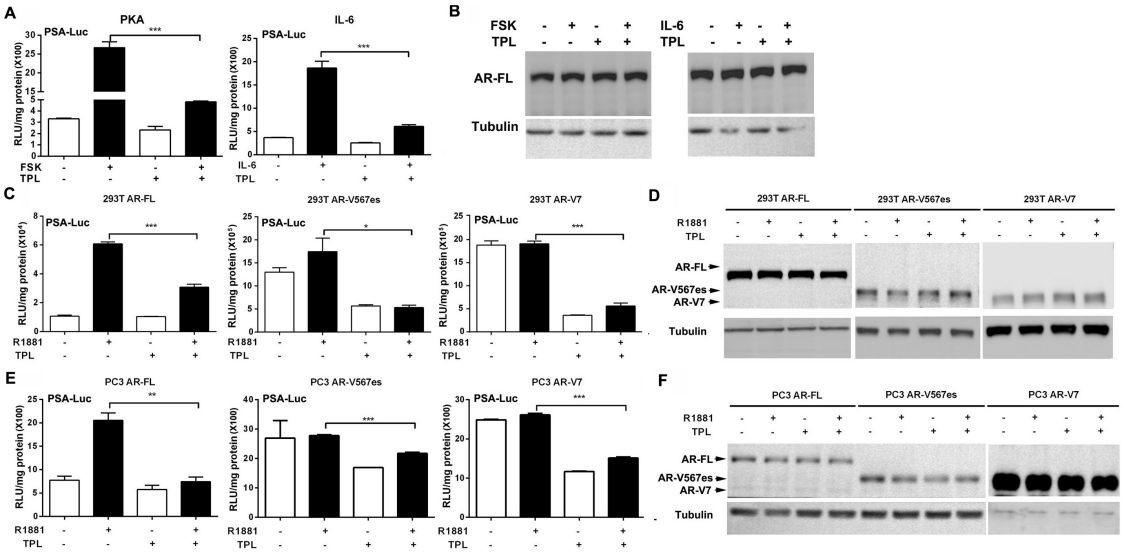
Extraction of cytoplasmic and nuclear proteins

C4-2R and parental C4-2 cells were cultured in phenol red-free RMPI-1640 medium containing 8% CSS for 3 days. Cells were harvested and washed twice with PBS, then fractionated into nuclear and cytosolic proteins with a Cytoplasmic/Nuclear Extraction kit (E221-01, Vazyme). Lamin B and Tubulin were used as the loading controls for nuclear and cytoplasmic fractions, respectively.

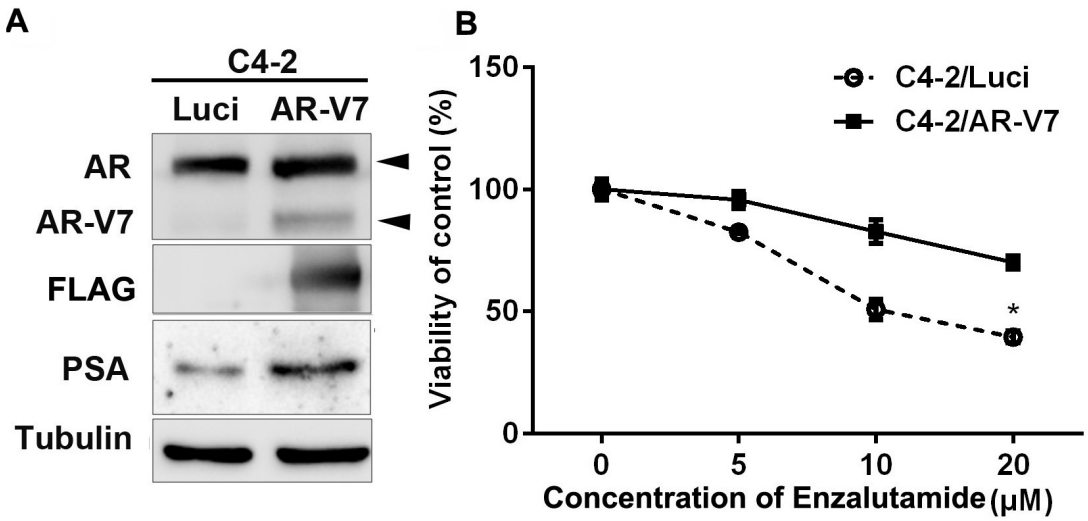
Biomolecular interaction analysis (BIA)

AR-FL and AR-AF1 his-fusion proteins were prepared as previously described.[3] The binding of TPL and AR was monitored by BIA based on the surface plasmon resonance (SPR) as previously described.[4]. In short, his-fusion AR proteins were immobilized on the CM5 chips through a his-tag capture procedure according to the manufacturer's protocol. TPL at different concentrations (6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM) in 5% DMSO and 1 x PBS were then injected over the activated surface at a rate of 15 μL/min at 25 °C. The signals were collected and evaluated by using the BIAcore T200 system (GE).

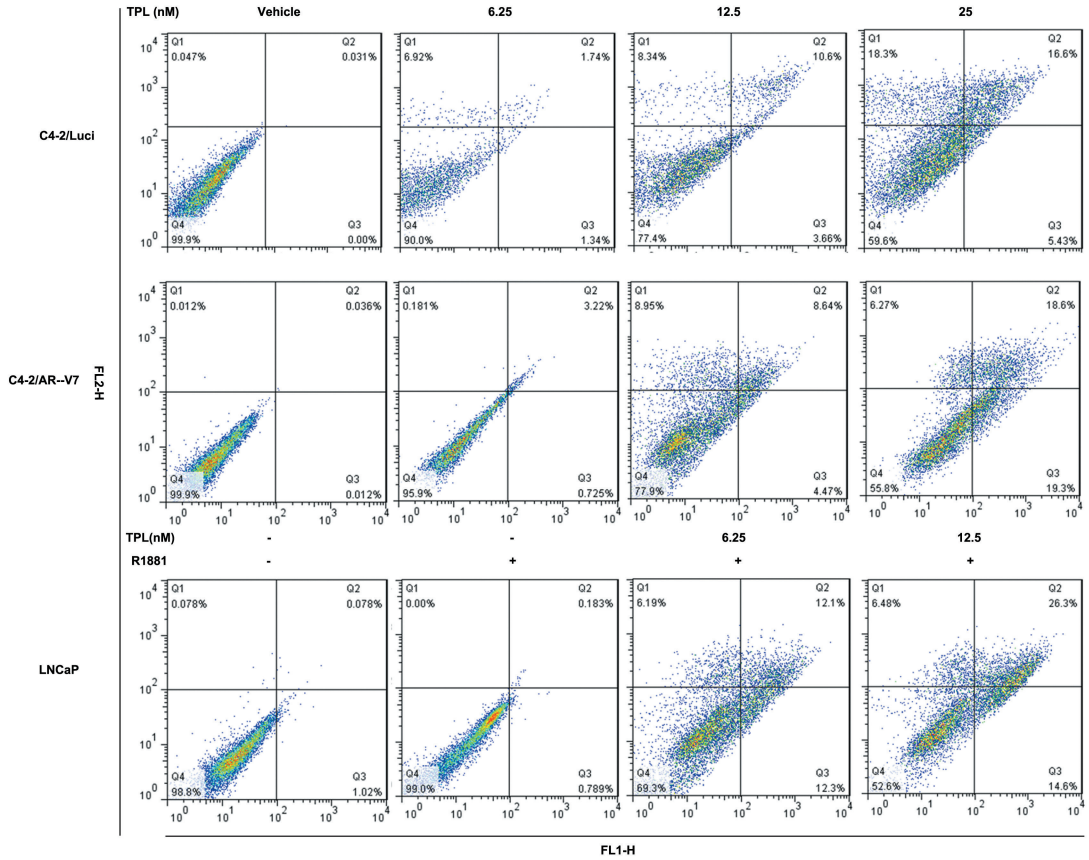
Supplementary figures and legends



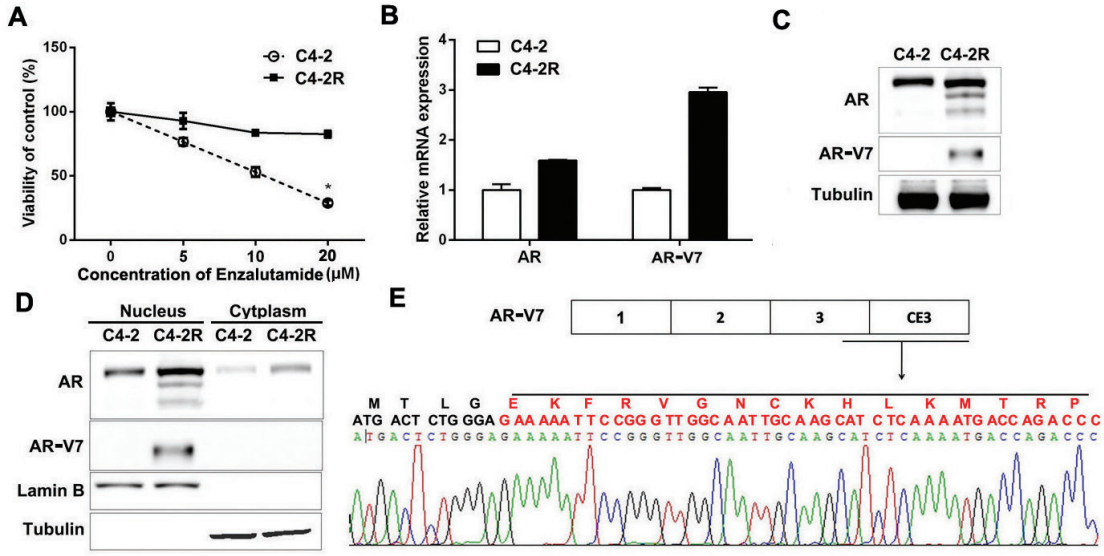
Supplementary Figure S1. TPL inhibits the ligand-independent activation of AR. (A) LNCaP cells were transfected with the PSA-luciferase reporter and treated with TPL and forskolin (FSK) / IL-6 under serum-free conditions for 24 h. (B) Western blot analysis of AR in cells extracts from (A). (C) 293T cells transfected with the PSA-luciferase reporter and plasmids expressing wild-type AR (AR-FL), AR-V7 or AR-V567es were treated with R1881 or TPL for 24 h. (D) The protein levels of AR and AR-Vs were detected in cells extracts from (C). (E) PC3 cells transfected with the PSA-luciferase reporter and plasmids expressing AR-FL, AR-V7 or AR-V567es were treated with R1881 or TPL. (F) Expression of AR and AR-Vs in cells extracts from (E).



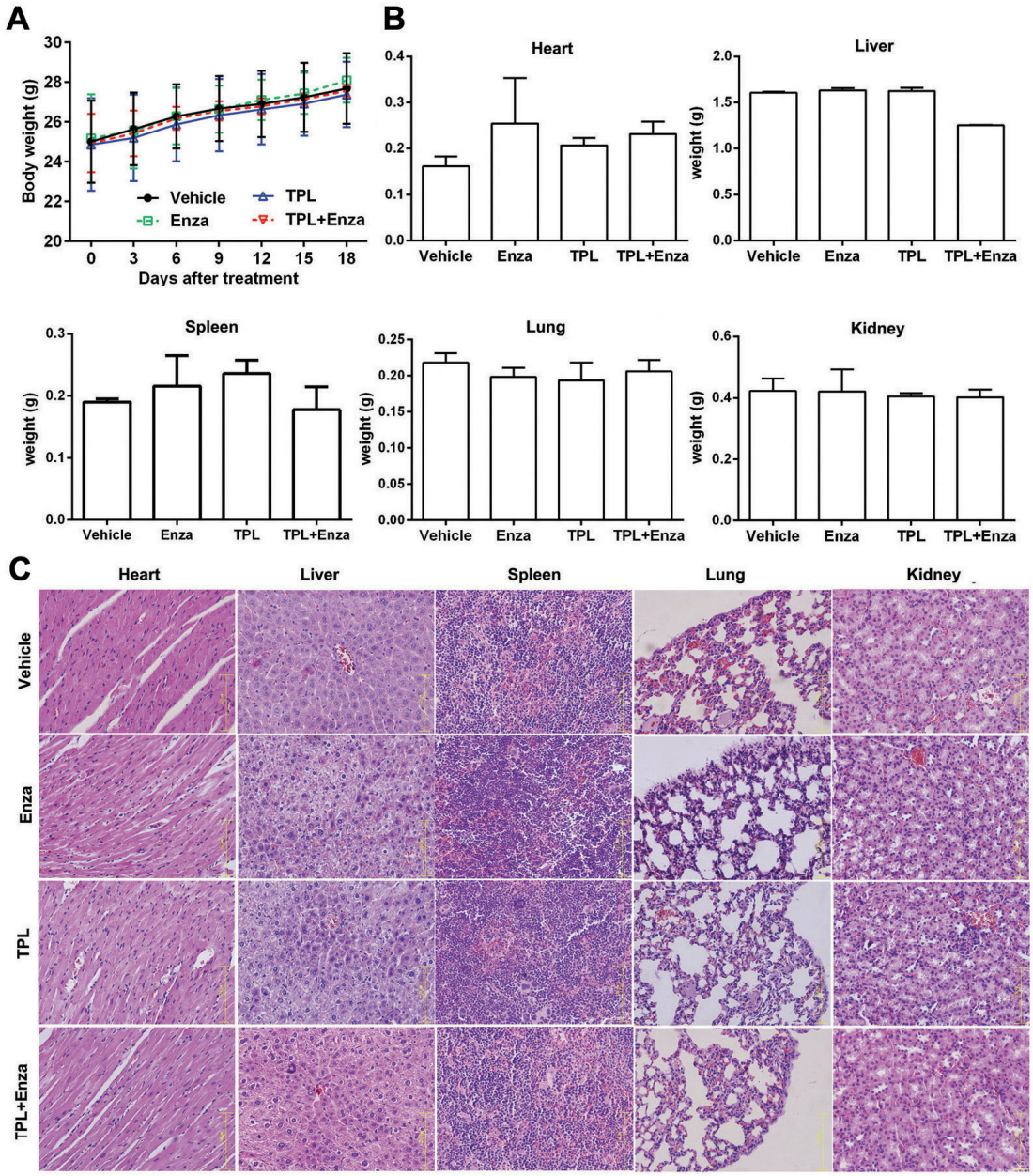
Supplementary Figure S2. Overexpression of AR-V7 in C4-2 cells. (A) C4-2/Luci and C4-2/AR-V7 cells were cultured in RPMI 1640 medium containing 8% CS-FBS for 48 h, and then indicated proteins were detected by western blotting. (B) C4-2/Luci and C4-2/AR-V7 cells were treated with indicated doses of enzalutamide for 72 h and detected by using MTT assays.



Supplementary Figure S3. The apoptosis induction effect of TPL on CRPC cells. C4-2/Luci, C4-2/AR-V7 and LNCaP cells were treated with various doses of TPL and incubated with AV-FITC (FL1-H) and PI (FL2-H). Stained cells were analyzed by FACS. The percentages of intact cells (AV-/PI−) and apoptotic cells at different stages (AV+/PI−, AV+/PI+ and AV-/PI+) are presented.



Supplementary Figure S4. Enzalutamide-resistant C4-2 cells (C4-2R) express AR variants. (A) C4-2 parental cells and C4-2R cells were treated with various doses of enzalutamide in RPMI 1640 medium containing 8% FBS for 48 h, and then MTT assays were performed. (B) AR-FL and AR-V7 mRNA levels in C4-2 parental cells and C4-2R cells were analyzed by qRT-PCR. (C) AR-FL and AR-V7 protein levels in C4-2 parental cells and C4-2R cells were analyzed by Western blotting. (D) AR-FL and AR-V7 protein levels in cytosolic and nuclear fractions of C4-2 parental cells and C4-2R cells were analyzed by Western blotting. Lamin B and tubulin were used as markers for the integrity of the nuclear and cytosolic fractions, respectively. (E) Sequencing data of AR-V7 in C4-2R cells.



Supplementary Figure S5. TPL has no apparent toxicity *in vivo*. (A) Body weights of mice in the different groups. (B) Weights of heart, liver, spleen, lung and kidney from mice in the different groups. Organs were harvested at the end of the experiment. (C) H&E staining of the above organs.

Table S1. Primer sets, subcloning primers and siRNA list

qPCR primer			
No.	**Gene**	**Direction**	**Sequence (5' to 3')***
1	AR	Forward	CAGTGGATGGGCTGAAAAAT
		Reverse	GGAGCTTGGTGAGCTGGTAG
2	AR-V7	Forward	CTACTCCGGA CCTTACGGGGACATGCG
		Reverse	TGCCAACCCGGAATTTTTCTCCC
3	PSA	Forward	ACGCTGGACAGGGGGCAAAAG
		Reverse	GGGCAGGGCACATGGTTCACT
4	FKBP5	Forward	AGGAGGGAAGAGTCCCAGTG
		Reverse	TGGGAAGCTACTGGTTTTGC
5	TMPRSS2	Forward	CAGGAGTGTACGGGAATGTGATGGT
		Reverse	GATTAGCCGTCTGCCCTCATTTGT
6	UBE2C	Forward	TGGTCTGCCCTGTATGATGT
		Reverse	AAAAGCTGTGGGGTTTTTCC
7	AKT1	Forward	TGGACTACCTGCACTCGGAGAA
		Reverse	GTGCCGCAAAAGGTCTTCATGG
8	β-actin	Forward	AATGTCGCGGAGGACTTTGAT
		Reverse	AGGATGGCAAGGGACTTCCTG
9	AR-V7-exon2	Forward	TGTCACTATGGAGCTCTCACATGTGG
	AR-V7-834	Reverse	CTGTGGATCAGCTACTACCTTCAGCTC
ChIP qPCR primer			
10	PSA-PR-ARE	Forward	CCTAGATGAAGTCTCCATGAGCTACA
		Reverse	GGGAGGGAGAGCT AGCACTTG
11	PSA-EN-ARE	Forward	TGGGACAACTTGCAAACCTG
		Reverse	CCAGAGTAGGTCTGTTTTCAATCCA
12	PSA-TATA	Forward	GTTGTCCAGCCTCCAGCAG
		Reverse	CCCTATAAAACCTTCATTCCCCA
XPB shRNA for subcloning in pLKO.1			
13	shXPB-1	Forward	CCGGCGACTGAACAAACCCTATATCCTCGAGGATATAGGGTTTGTTCAGTCGTTTTTG
		Reverse	AATTCAAAAACGACTGAACAAACCCTATATCCTCGAGGATATAGGGTTTGTTCAGTCG
14	shXPB-2	Forward	CCGGGATCCGAGAATGCCGCTTAAGCTCGAGCTTAAGCGGCATTCTCGGATCTTTTTG
		Reverse	AATTCAAAAAGATCCGAGAATGCCGCTTAAGCTCGAGCTTAAGCGGCATTCTCGGATC
siRNA oligonucleotides			
15	siNC		UUCUCCGAACGUGUCACGUTT
16	siXPB		GCACCAAAGUGGAUGAAUATT

References

1. Huang W, He T, Chai C, *et al*. Triptolide inhibits the proliferation of prostate cancer cells and down-regulates SUMO-specific protease 1 expression. PLoS One. 2012; 7: e37693

2. Houghton P, Fang R, Techatanawat, *et al*. The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. Methods. 2007; 42: 377-87.

3. Reid J, Kelly SM, Watt K, *et al*. Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation. Influence of structure-stabilizing solutes and protein-protein interactions. J Biol Chem. 2002; 277: 20079-86.

4. Beerheide W, Bernard HU, Tan YJ, *et al*. Potential drugs against cervical cancer: zinc-ejecting inhibitors of the human papillomavirus type 16 E6 oncoprotein. J Natl Cancer Inst. 1999; 91:1211-20.