Supplementary Data and Methods

A novel small molecule inhibits tumor growth and synergizes effects of enzalutamide on prostate cancer

Jiongjia Cheng, Stephanie Moore, Jorge Gomez-Galeno, Dong-Hoon Lee, Karl J. Okolotowicz and John R. Cashman

Human BioMolecular Research Institute and ChemRegen, Inc., San Diego, CA, 92121, USA

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Chemicals and Reagents

The following materials (purchased from) were used: Dulbecco's phosphate buffered solution (DPBS), trypsin-EDTA, RPMI (Corning Inc., Corning, NY); Fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA); SYBR Green I nucleic acid stain in DMSO (Lonza, Allendale, NJ); protease inhibitors (Roche, Indianapolis, IN); Pierce BCA protein assay kit and ECL reagent (Thermo Scientific, Rockford, lL); NuPAGE® Bis-Tris Gel (Life Technologies, Carlsbad, CA); Enzalutamide, Abiraterone (eNovation Chemicals, Bridgewater Township, NJ); Triton-X-100, bovine serum albumin fraction V (BSA), EDTA, sodium chloride, nonylphenoxypolyethoxylethanol (NP-40), 3α,12α-dihydroxy-5β-cholanic acid sodium salt (sodium deoxycholate), polyethylene glycol sorbitan monolaurate (Tween-20), dodecyl sulfate sodium salt (SDS) (Sigma-Aldrich, St. Louis, MO); and other chemicals, reagents, solvents and devices (VWR, San Diego, CA). RIPA buffer: 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium doxycholate, 0.1 % SDS in the presence of 1× protease inhibitors.

Antibodies

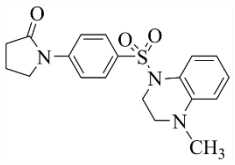
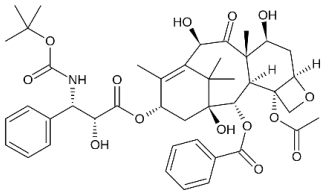
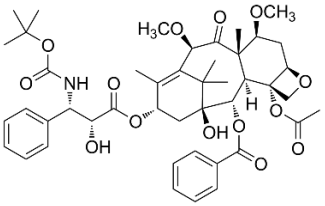
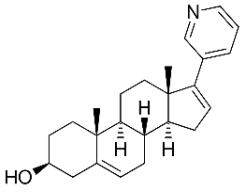
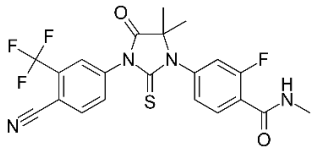
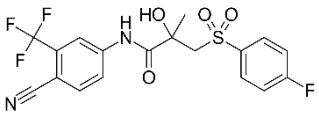
The following antibodies were used: anti-p53 (Cell Signaling #9282; Bio-Rad, #MCA1701), anti-phospho-Ser15-p53 (Cell Signaling #9284), anti-phospho-Ser428-ATR (Cell Signaling #2853), anti-phospho-Ser1981-ATM (Cell Signaling #5883), anti-Bcl-2 (Santa Cruz #sc-509), anti-Bcl-xL (Cell Signaling #2764; Santa Cruz #sc-8392), anti-Mcl-1 (Santa Cruz #sc-74436), anti-Bax (Cell Signaling #5883), anti-Bak (Santa Cruz #sc-517390), anti-Cytochrome c (Santa Cruz #sc-13156), anti-PARP (Cell Signaling #9532), anti-Tom20 (Santa Cruz #sc-17764), anti-VDAC1 (Santa Cruz #sc-390996), anti-HSP60 (Santa Cruz #sc-13115), anti-acetylated-tubulin (Sigma #T7451), anti-GAPDH (Santa Cruz #sc-47724) and anti-HSP90a/ß Antibody (Santa Cruz #sc-13119).

Table S1. Clinical chemistry serum values observed for vehicle-, enzalutamide (Enza)-, PAWI-2 and both enzalutamide and PAWI-2-treated mice

Condition	Parameter[a]							
	ALP[b] (U/L)	SGPT (ALT)[b] (U/L)	SGOT (AST)[b] (U/L)	Albumin (g/dL)	BUN[b] (mg/dL)	Creatinine (mg/dL)	Cholesterol (mg/dL)	Glucose (mg/dL)
Vehicle	59	65	127	2.8	22	0.2	126	175
Enza	48	130	413	2.3	24	0.1	102	157
PAWI-2	46	168	253	2.2	24	0.2	120	84
Enza + PAWI-2	38	75	128	2.0	32	0.1	125	82

[a]Independent analysis of serum samples from four different animal groups (vehicle-, enzalutamide (Enza)-, PAWI-2 and both enzalutamide and PAWI-2-treated mice);
[b]ALP: Alkaline Phosphatase; SGPT (ALT): Serum Glutamic Pyruvic Transaminase (Alanine Aminotransferase); SGOT (AST): Serum Glutamic Oxaloacetic Transaminase (Aspartate Aminotransferase); BUN: Blood Urea Nitrogen.

Table S2. Comparison of pharmaceutical properties of PAWI-2 to other PCa drugs.

Drug name	**Structure**[a]	**Lipinski value**[a]	**ADME data**[a,b]	**Safety/ Toxicity**[a,c]
PAWI-2		0	Bioavailability = 12% (HCl salt; rat); Vss, N/A; $t_{1/2}$ = 16.5 h; CL = 43 L/min/kg (rat); ~5% metabolized	No toxicity observed at 1 g/kg (i.p.; rat)
Docetaxel		2	Bioavailability = ~8%; Vss= 113 L; $t_{1/2}$ = 11.1 h; CL = 21 L/h; hepatically metabolized to 1 major and 3 minor metabolites	LD_{50} >2 g/kg (oral; rat)
Cabazitaxel		3	Bioavailability = 7.7%; Vss= 4864 L; $t_{1/2}$ = 13.5 h; CL = 48.5 L/h; extensively metabolized	LD_{50} = 500 mg/kg (oral; rat)
Abiraterone		1	Bioavailability = ~10% (acetate salt); Vss= 19669 L; $t_{1/2}$ = 10.3 h; CL = 307 L/h; metabolized to 2 inactive major metabolites	LD_{50} = 980 mg/kg (oral; rat)
Enzalutamide		0	Bioavailability = 89.7% (rat); Vd/F= 110 L; $t_{1/2}$ = 5.8 days; CL = 0.56 L/h; hepatically metabolized into active metabolite	LD_{50} = 400 mg/kg (oral; mouse)
Bicalutamide		0	Bioavailability unknown, highly absorbed; Vss, N/A; $t_{1/2}$ = 5.9 days; CL = 0.32 L/h; stereoselective metabolism	LD_{50} >2 g/kg (oral; rat)

[a]Structure, Lipinski values and ADME, Safety/Toxicity data of listed clinical PCa drugs were obtained from Drugbank website (https://www.drugbank.ca/). The data of PAWI-2 was cited from previously published paper [Cheng et al., *Cancer Res*., 2018, 78, 5072].

[b]Vss, volume of distribution at steady state; $t_{1/2}$, the mean terminal elimination half-life; CL, clearance; Vd/F, apparent volume of distribution; N/A, not available;

[c]LD_{50}: lethal dose for 50 percent of the animals tested.

Figure S1. Effect of PAWI-2 on **A**) inhibition of cell viability (72 hours), **B**) activation of cell apoptosis (24 hours) in 11220-hTERT normal prostate epithelia cells; data for prostate cancer cells (PC-3 and LNCaP) also shown. The dose of PAWI-2 was 0.32 nM to 5 μM. Data are mean ± SD (n = 3); *P*-values were estimated by one-way ANOVA test (**P*<0.05, ***P*<0.01).

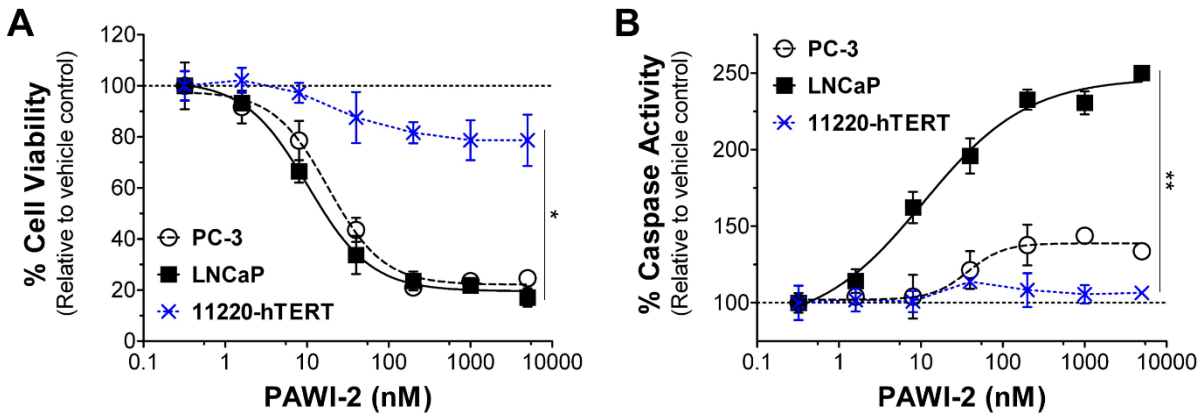


Figure S2. Effect of PAWI-2 on cell viability of PCa cells in the presence of enzalutamide in PC-3 and LNCaP cells. Concentration of enzalutamide was 500 nM. The concentration of PAWI-2 was 20 and 100 nM, as indicated. Treatment time was 24 hours. Data represents the mean ± SD (n = 3) as determined for compound-treated samples relative to vehicle control. Veh, vehicle control (0.5% DMSO); Enza, enzalutamide. There was no significant difference as determined by one-way ANOVA test (*P*>0.05).

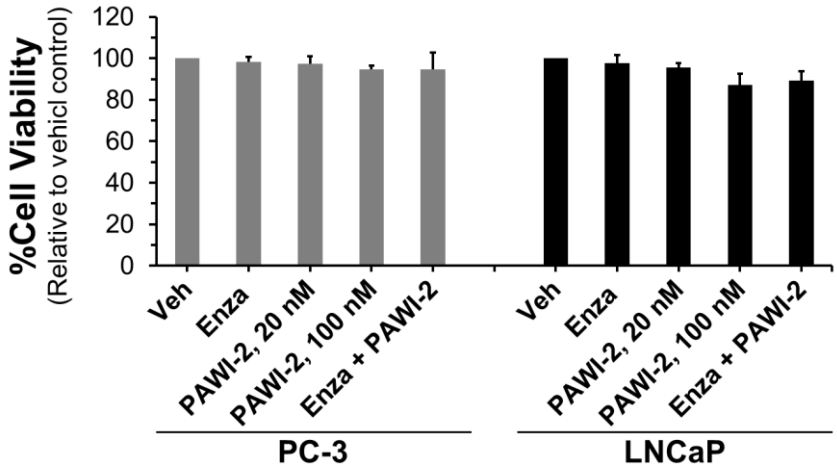


Figure S3. Effect on prostate cancer cell viability for PAWI-2 (concentration <IC_{50}) in combination with enzalutamide or abiraterone in **A**) PC-3 and **B**) LNCaP PCa cells. The concentration of PAWI-2 was 8 and 2 nM in PC-3 and LNCaP cells, respectively; Enza, enzalutamide; Abi, abiraterone; vehicle control (0.5% DMSO). Data are mean ± SD (n = 3); *P*-values were estimated by one-way ANOVA test (****P*<0.001).

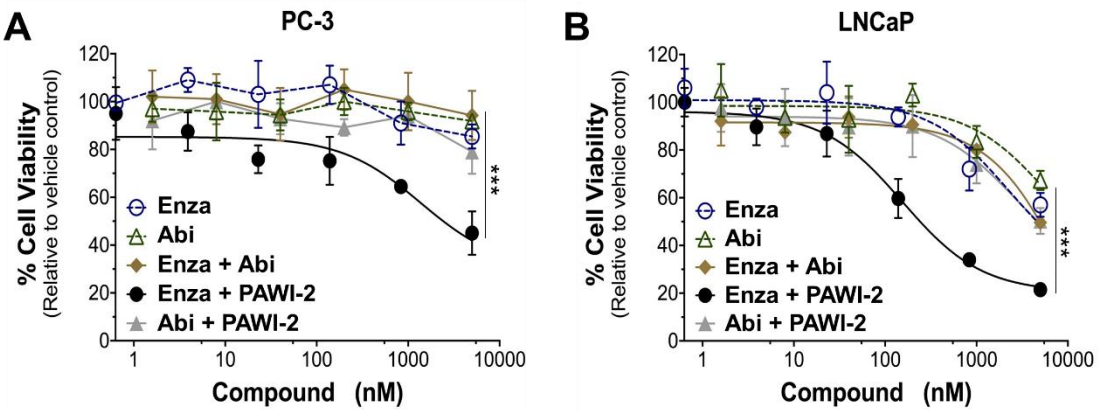


Figure S4. Time-dependent activation of Caspase-3/7 by PAWI-2 in the presence of enzalutamide (Enza) and/or abiraterone (Abi) in **A**) PC-3 and **B**) LNCaP cells. Concentrations used: PC-3 (Enza, 500 nM; Abi, 3 µM; PAWI-2, 100 nM); LNCaP (Enza, 500 nM; Abi, 3 µM; PAWI-2, 20 nM). Treatment time was 0-72 hours. Veh, vehicle control (0.5% DMSO); Enza, enzalutamide; and Abi, abiraterone. Data represents the mean ± SD (n = 3) as determined for compound-treated samples relative to vehicle control. *P*-values were estimated by one-way ANOVA test (***P*<0.01, ****P*<0.001).

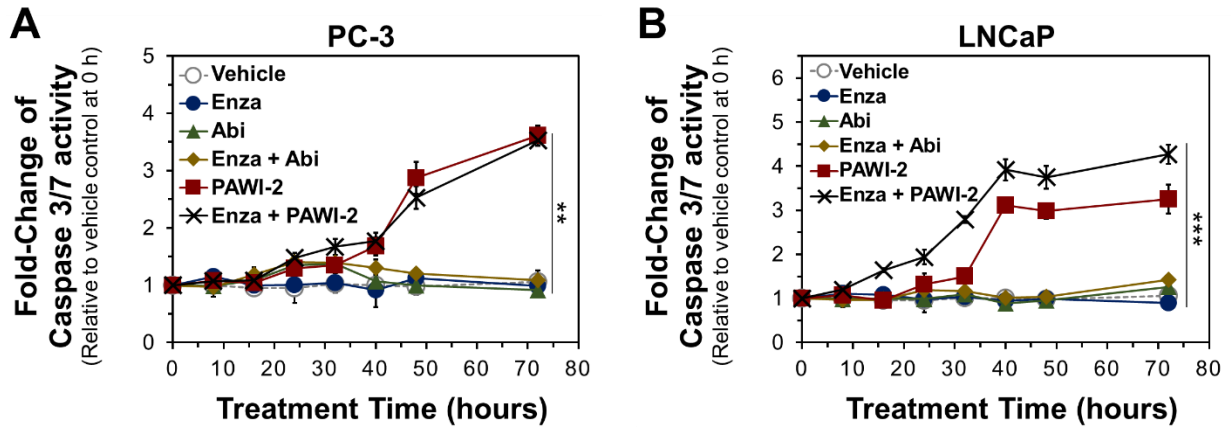


Figure S5. Effect of PAWI-2 on DNA damage pathway marker (phosphorylated-ATR), apoptotic pathway markers (p53, phosphorylated p53 (p53), PARP cleavage). **A**) Western blots and **B**) densitometry analysis were determined from whole-cell extracts of PC-3 and LNCaP cells in the presence of enzalutamide and/or abiraterone. The concentrations used: PC-3 (Enza, 500 nM; Abi, 3 µM; PAWI-2, 100 nM); LNCaP (Enza, 500 nM; Abi, 3 µM; PAWI-2, 50 nM). Treatment time was 48 hours for PARP and 24 hours for other markers; Veh, vehicle control (0.5% DMSO); Enza, enzalutamide; and Abi, abiraterone. GAPDH was used as an internal control. Data are mean ± SD (n = 3) in **B**.

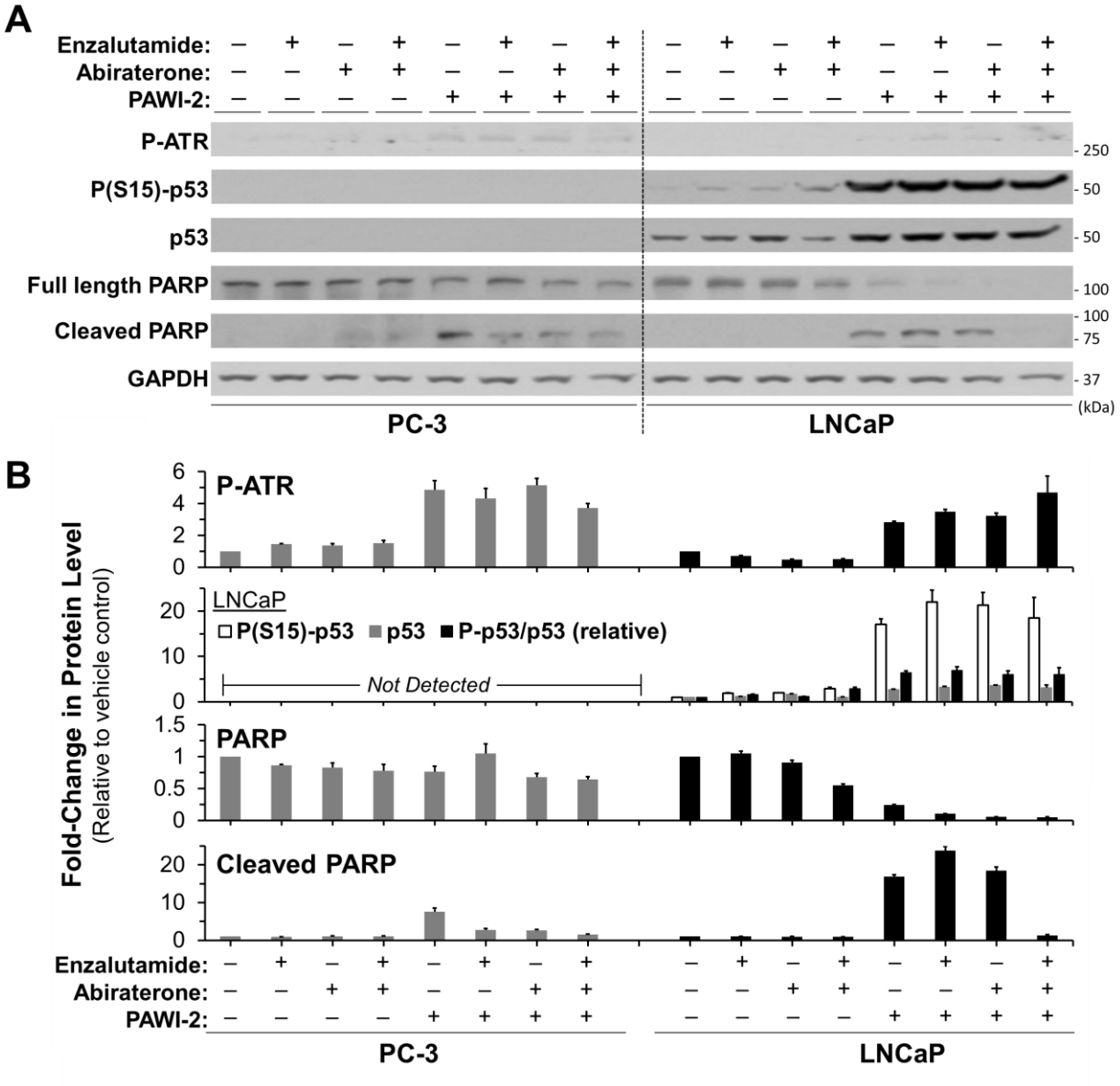


Figure S6. Time-dependent effect on mitochondrial membrane potential by PAWI-2 in the presence of enzalutamide and/or abiraterone determined by TMRE assay in **A**) PC-3 and **B**) LNCaP cells. Concentrations used: PC-3 (Enza, 500 nM; Abi, 3 µM; PAWI-2, 100 nM); LNCaP (Enza, 500 nM; Abi, 3 µM; PAWI-2, 50 nM). Treatment time was 0-48 hours. Veh, vehicle control (0.5% DMSO); Enza, enzalutamide; and Abi, abiraterone. Data are mean ± SD (n = 3). *P*-values were estimated by one-way ANOVA test (**P*<0.05, ***P*<0.01).

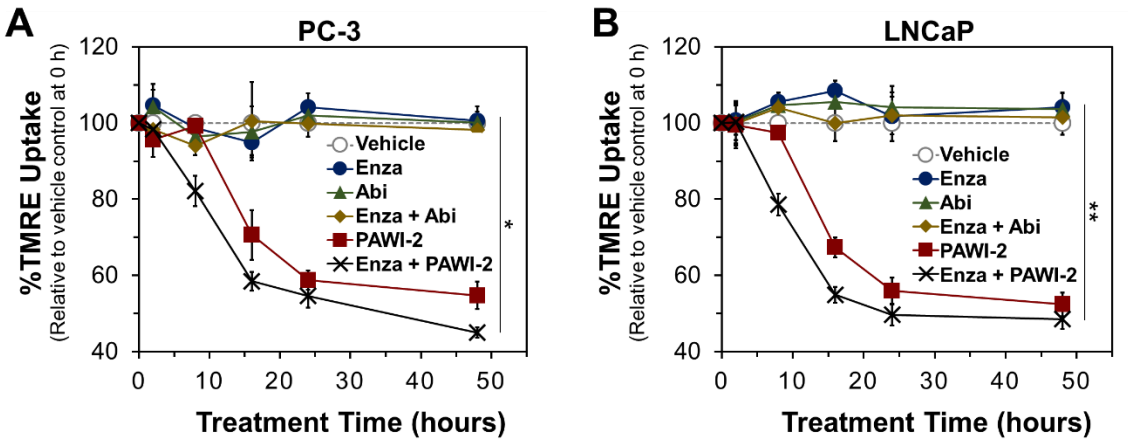


Figure S7. Western blot analysis of the effect of PAWI-2 on the inhibition of acetylated tubulin (Ac-Tub) as determined by **A**) dose-dependence of PAWI-2 and **B**) in the presence of enzalutamide in whole cell extracts of PC-3 and LNCaP cells. Concentrations of PAWI-2 were 20, 50, 100 and 200 nM in **A** and 100 nM for PC-3 and 50 nM for LNCaP in **B**; Concentrations of enzalutamide and abiraterone treatment were 500 nM and 3 µM, respectively. Treatment time used was 24 hours. Veh, vehicle control (0.5% DMSO); Ac-Tub, acetylated tubulin. GAPDH was used as an internal control.