Figure S1. Representative western blots. 49C^{ENZR}, 49C ENZ-resistant; 49F^{ENZR}, 49F ENZ-resistant; AR, androgen receptor; ENZ, enzalutamide; PSA, prostate-specific antigen; Vin, vinculine.

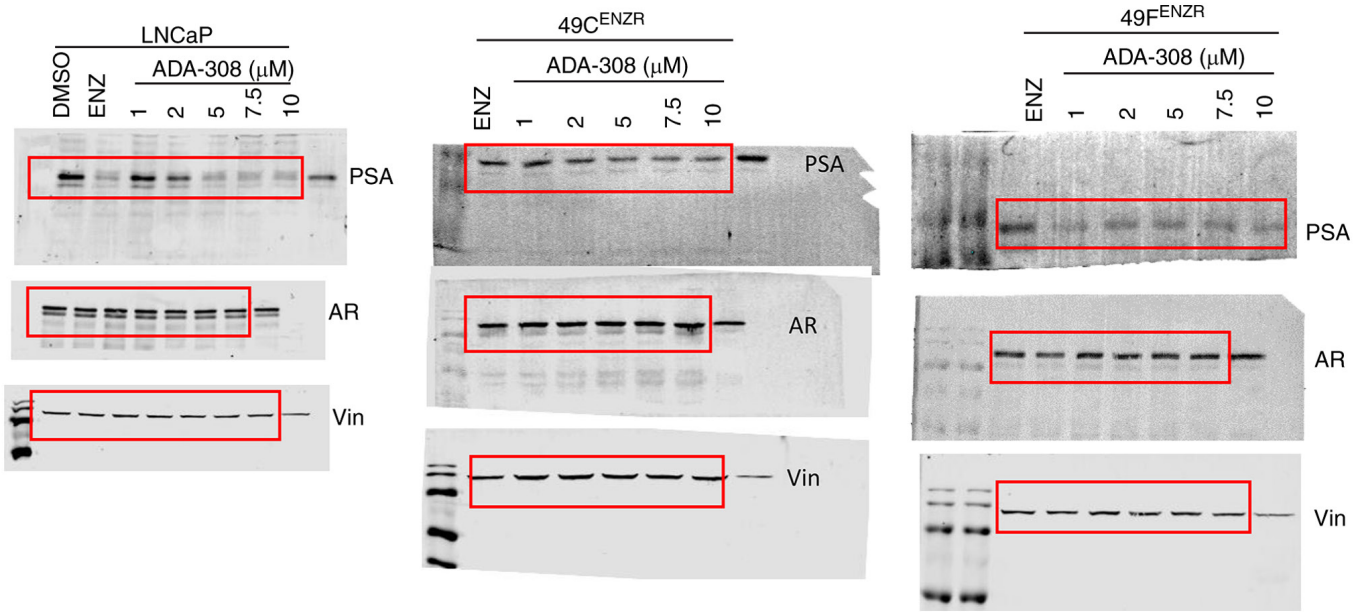

Figure S2. Representative flow cytometry histograms. The LNCaP, 49F^{ENZR} and 49C^{ENZR} cell lines were treated with DMSO, ENZ (10 μM) or ADA-308 (10 μM) for 72 h, and the cell cycle was measured using flow cytometry (n=3 independent biological replicates). 49C^{ENZR}, 49C ENZ-resistant; 49F^{ENZR}, 49F ENZ-resistant; ENZ, enzalutamide.

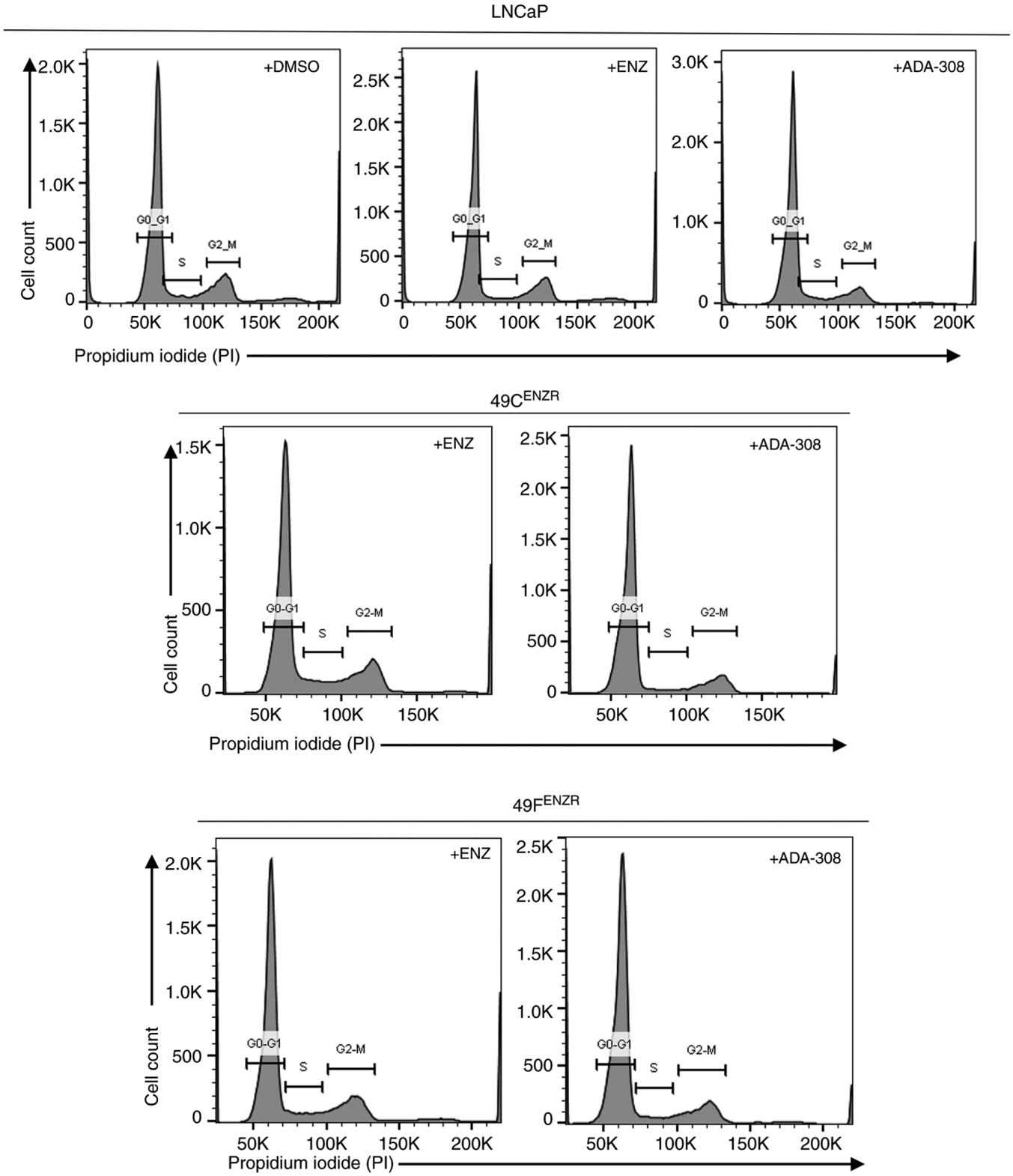

Figure S3. ADA-308 decreases AR activity in ENZ-resistant prostate cancer cell lines. (A) Western blot shows that treatment with ADA-308 inhibits PSA expression in ENZ-resistant $49C^{ENZR}$ in a dose-dependent manner (1-10 μM); 10 μM ENZ was used as the control. Cells were treated for 72 h before protein lysate was harvested (n=3 independent biological replicates). Vin was used as a loading control. (B) ADA-308 decreases the transcription of AR target genes in $49C^{ENZR}$ cells, as shown by RT-qPCR. Cells were treated for 72 h with 1-10 μM ADA-308 or 10 μM ENZ before RNA was extracted (n=3 independent biological replicates). The bar graph shows the mean ± standard deviation. Data were analyzed using a one-way ANOVA to assess the variance between dosages. Post hoc comparisons were performed using Dunnett's test to compare each treatment group to ENZ. (C) ADA-308 at 10 μM inhibits AR transactivation, as measured by luciferase assay with R1881-induced activation of Probasin AR reporter in $49C^{ENZR}$ cells (n=2 independent biological replicates). Data shows relative fluorescence normalized to ENZ. Data were analyzed using a one-way ANOVA to assess the variance between dosages. Post hoc comparisons were performed using Dunnett's test to compare each group to ENZ. (D) RT-qPCR results shows the relative mRNA expression of AR and PSA in LNCaP cells treated with ENZ (10 μM), ODM-201 (10 μM) or ADA-308 (10 μM) for 72 h. ****P<0.0001. All exact P-values are listed in Table S1. $49C^{ENZR}$, 49C ENZ-resistant; AR, androgen receptor; ENZ, enzalutamide; PSA, prostate-specific antigen; Vin, vinculine.

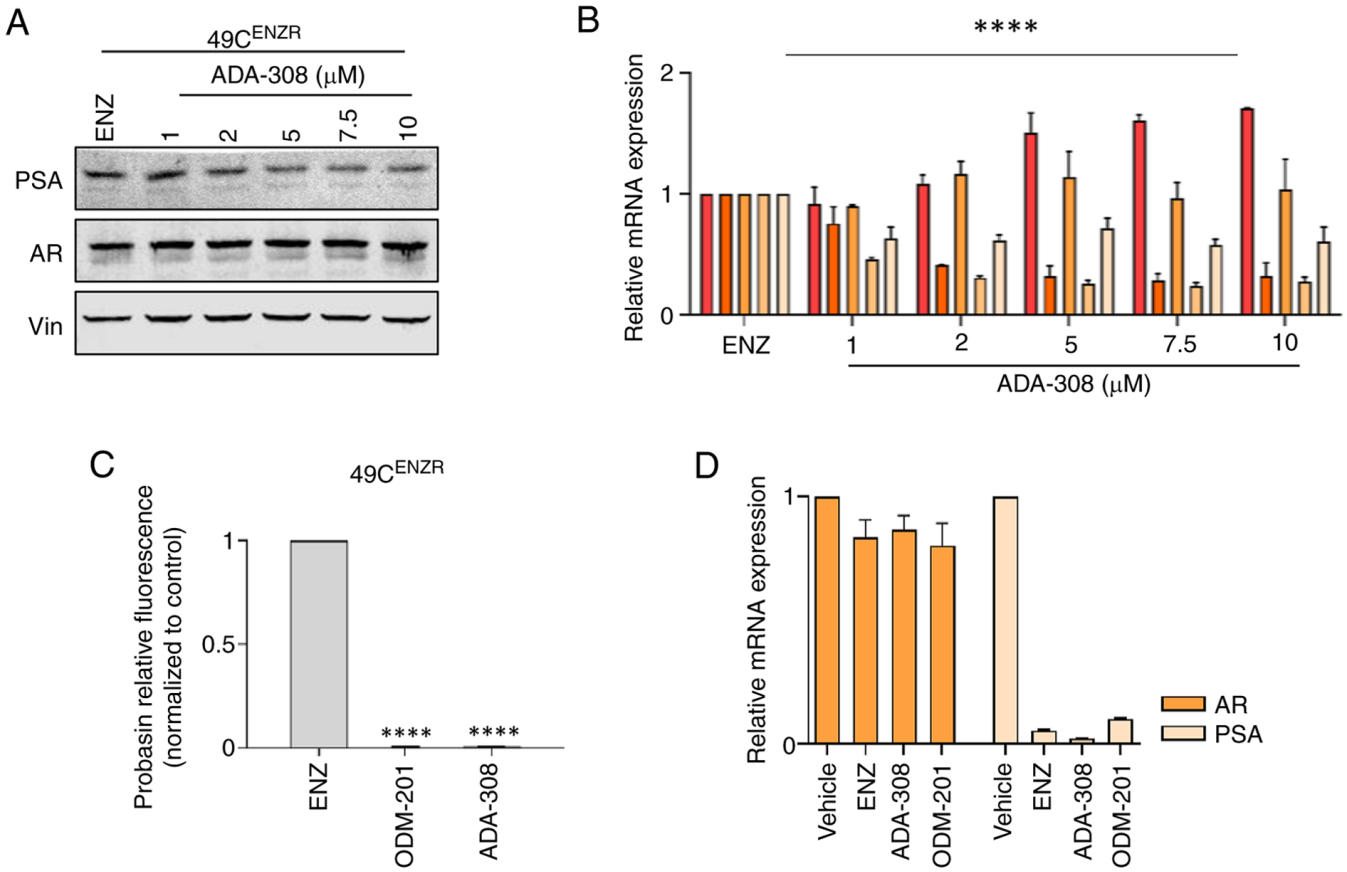

Figure S4. ADA-308 inhibits AR nuclear translocation, induces G1 accumulation and inhibits cell proliferation in ENZ-resistant prostate cancer cell lines *in vitro*. (A) Immunofluorescence of 49C^{ENZR} cells treated with DMSO (control), AR antagonists ENZ (10 μM) or ADA-308 (10 μM) for 24 h, with or without a 20-min R1881 (10 nM) treatment. Scale bar, 10 μm; AR is shown in green and DAPI is show in blue. (B) 49C^{ENZR} (top) and 49F^{ENZR} (bottom) cell lines were treated with ENZ (10 μM), ODM-201 (10 μM) or ADA-308 (10 μM) for 7 days, and proliferation was measured using IncuCyte, reported as a confluence ratio over day 0. Data are shown as the mean ± standard deviation, with significance evaluated at the endpoint using one-way ANOVA followed by Dunnett's multiple comparisons test (n=3 independent biological replicates). (C) 4C^{ENZR} cell line was treated with ENZ (10 μM) or ADA-308 (10 μM) for 72 h; cell cycle was measured using flow cytometry (Two-tailed unpaired t-test; 49C^{ENZR} ADA-308 vs. CTL: G0/G1, P=0.004; S, P=0.05; G2/M, P=0.06); n=3 independent biological replicates). See Figure S2 for the representative histograms. All exact P-values are listed in Table SI. 49C^{ENZR}, 49C ENZ-resistant; 49F^{ENZR}, 49F ENZ-resistant; AR, androgen receptor; ENZ, enzalutamide.

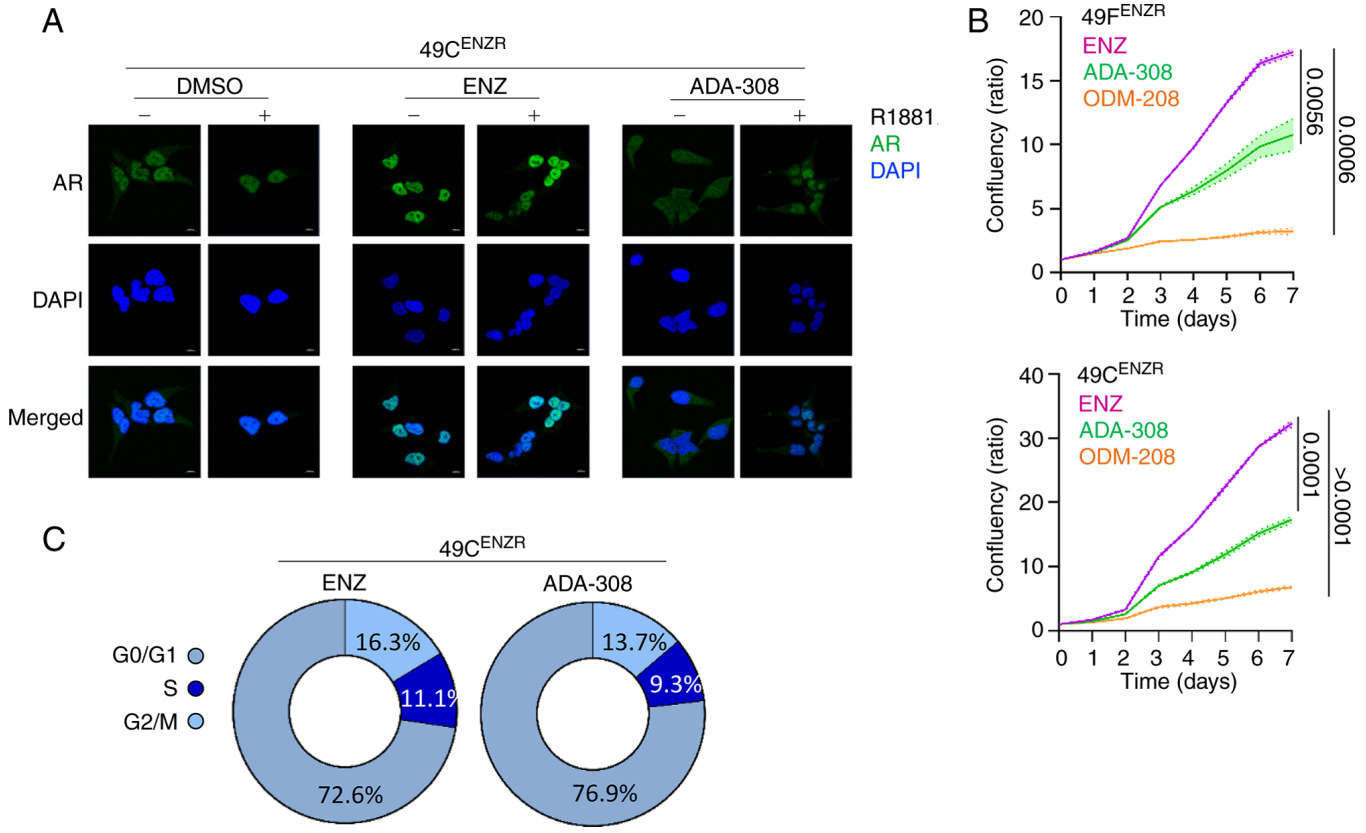

Figure S5. ADA-308 reduces tumour growth in ENZ-resistant cells *in vivo*. (A) *In vivo* study treatment groups, number of mice recruited to each group and respective doses. (B) Castrated Nu/Nu mice were inoculated with bilateral 49C^{ENZR} tumors, and tumor dimensions were measured biweekly. Mice were assigned to vehicle group 1, vehicle group 2, continuum on ENZ (10 mg/kg), two doses of ADA-308 (12.5 mg/kg and 25 mg/kg) or ODM-201 (50 mg/kg). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test, comparing the last time point of treatment to the vehicle control. (C) Tumor volume (mm^3) for individual mice for each treatment group at 3 weeks of treatment. All exact P-values and raw data are listed in Table SIII. 49C^{ENZR}, 49C ENZ-resistant; Bid, twice a day; ENZ, enzalutamide; qd, four times a day.

A

Study Groups	N	Dose p.o.
Vehicle 1	5	Qd
Vehicle 2 (ODM)	5	Bid
ENZ	11	10mg/kg qd+ veh
ADA-308	10	12.5 mg/kg bid
ADA-308	12	25 mg/kg bid
ODM-201	11	50 mg/kg bid