# Drug repurposing approach for developing novel therapy for castration resistant prostate cancer

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## ABSTRACT

**Background:** Androgen deprivation therapy (ADT) is the prevalent first line therapy against advanced prostate cancer as an adjuvant for locally treating the high-risk disease. Malignant cells initially respond to ADT, but subsequently colonize and re-emerge as more aggressive phenotype known as castration resistant prostate cancer (CRPC). The second generation androgen receptor (AR) antagonist enzalutamide (ENZU) exhibits survival advantage in CRPC patients, however ~30% of patients develop resistance overtime activating AR in these tumors. These resistant tumors poorly respond to chemotherapy and acquire resistance partly due to enhanced aerobic glycolysis and biomass production known as the Warburg effect. Therefore, identification of an effective low-cost therapeutic alternative with fewer side effects may lead to increased survival and greatly benefit patient quality-of-life. Drug repurposing has emerged as a new option for overcoming resistance to chemotherapy. Previous studies from our laboratory (Mol. Cancer Ther. 13:2288-302, 2014) has demonstrated that synergistic combination of simvastatin (SIM), a drug for the treatment of hypercholesterolemia and metformin (MET), a glucose lowering drug inhibits CRPC growth, invasiveness and migration potential with minimal effect on normal prostate epithelial cells. Here we investigate whether combination of SIM and MET could be effective in the treatment of ENZU-resistant prostate cancer cells.

**Methods:** Human CRPC cells C4-2B-ENZU (C4-2B enzalutamide resistant) were generated by growing C4-2B cells in progressive concentration of ENZU 5-20μM to develop resistance and maintained in 5μM ENZU in the culture medium for >20 generations. 22Rv1 cells are inherently resistant to ENZU. These cells were treated with SIM and MET individually and in combination, followed by assessment of cell viability, crystal violet assay, cell cycle analysis, migration, invasion and expression of various target genes by Western blotting.

**Results:** C4-2B-ENZU and 22Rv1 cells were treated individually and in a combination of SIM and MET at pharmacological dose range (500nM-4μM SIM and 250μM-2mM MET). Combination treatment with 4μM SIM and 2mM MET (SIM+MET) led to significant and synergistic inhibition of cell viability, migration, invasion and cell cycle blockade in both cell lines. The individual treatments of SIM and MET exhibited little or no effect on these cells. Furthermore, SIM+MET combination decreased the expression of AR, AR-V7, p-Akt (Ser473), p-AMPK$\alpha_1/\alpha_2$ (Ser-485/491) and simultaneously increased p-AMPKα1 (Thr-172) and AMPKα kinase activity in these cells.

**Conclusion:** Combined action of SIM and MET may be an effective regimen for treatment of ENZU-resistant tumors. This opens new therapeutic modality for castration-resistant prostate cancer patients.

## MATERIALS & METHODS

Human CRPC cells C4-2B-ENZU (C4-2B enzalutamide resistant) were generated by growing C4-2B cells in 5-20μM of ENZU over 60 days and maintained in 5μM ENZU in the culture medium for 20 generations. 22Rv1 cells are inherently resistant to ENZU. The cells were treated with SIM (4μM) and MET (2mM) individually and in combination, followed by assessment of cell viability, crystal violet assay, cell cycle analysis, migration, invasion and expression of various target genes by Western blotting.

## RESULTS

### Combination SIM and MET inhibit cell proliferation

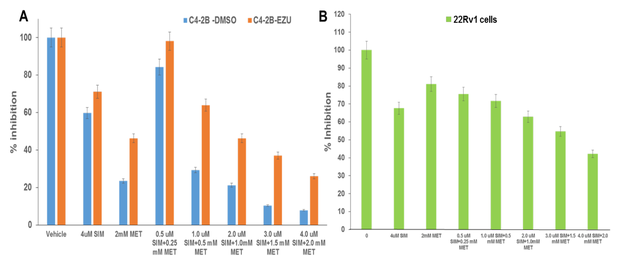


**Figure 1.** ***A.*** Individual effect of simvastatin (SIM) and metformin (MET) in enzalutamide (ENZU) resistant C4-2B cells and its parental counterpart ***B.*** 22Rv1 cell viability assay. Fraction by CompuSyn analysis revealed the combination effect of SIM+MET was higher in both cell lines. Values represent Mean ± SD.

### Combination SIM and MET inhibit cell growth

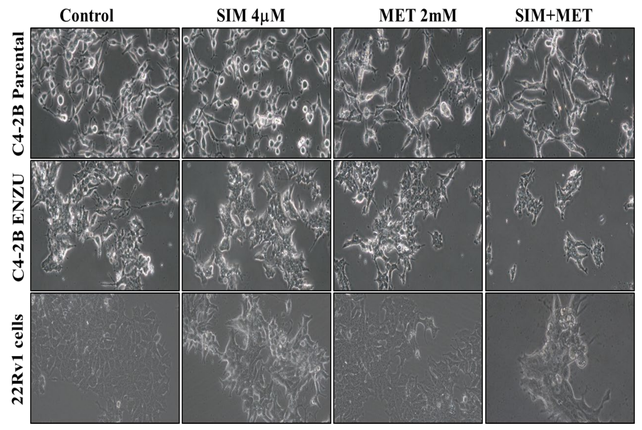


**Figure 2**: . Microscopic image of ***A.*** C4-2B-ENZU resistant cells and parental counterpart ***B.*** 22Rv1 cells treated with SIM and MET and its combination at the same concentration as shown. Magnification 20X.

### Combination SIM and MET induce cytotoxicity decreasing cell viability in 22Rv1 cells

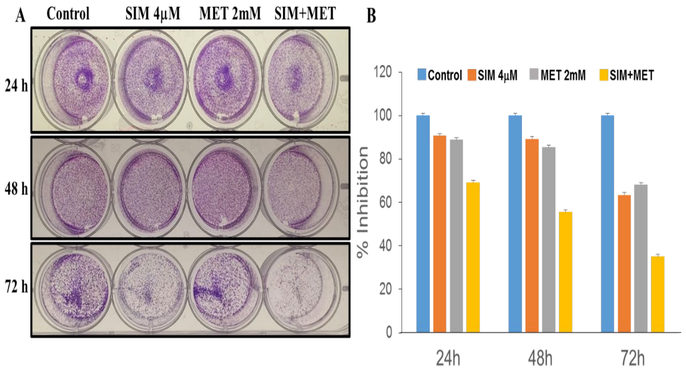


**Figure 3**. **A**. Photograph cell viability and ***B.*** percent cell growth inhibition in 22Rv1 cells. Individual effect of simvastatin (SIM) and metformin (MET) 22Rv1 cells. Values represent Mean ± SD.

### Combination SIM and MET induce cytotoxicity decreasing cell viability in C4-2B ENZU-resistant cells and parental cells

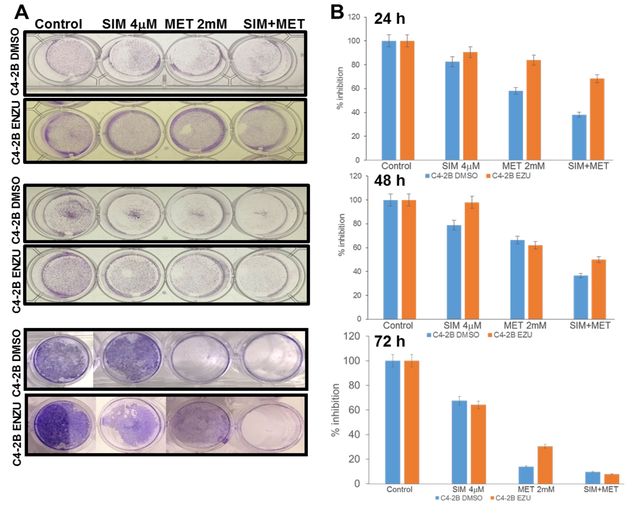


**Figure 4:** Representative image of cell viability assay using methylene blue and ***B.*** % inhibition after treatment of C4-2B enzalutamide-resistant cells and its parental counterpart with simvastatin (SIM), metformin (MET) and their combination (SIM+MET). Values represent Mean ± SD of 6-8 wells.

### Combination SIM and MET inhibits cell migration in CRPC cells

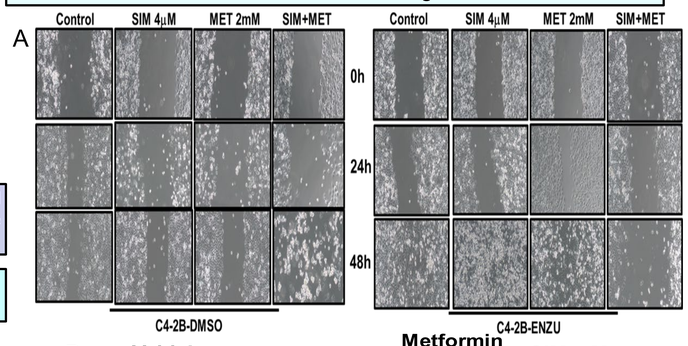


**Figure 5:** Migration assay in (A) enzalutamide-resistant C4-2B cells and its parental counterpart (B) 22Rv1 after treatment with simvastatin (SIM), metformin (MET) and their combination (SIM+MET).

### Combination SIM and MET inhibits invasion in CRPC cells

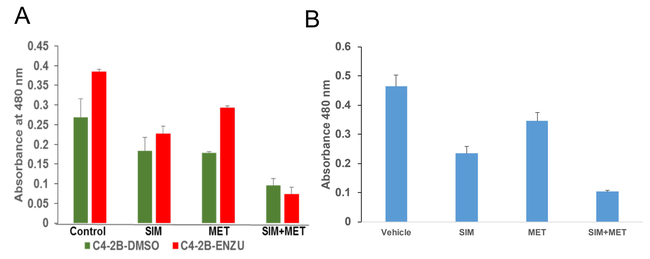


**Figure 6**: . Invasion assay in (A) enzalutamide-resistant C4-2B cells (C4-2B-ENZU) and its parental counterpart (B) 22Rv1 cells after treatment with simvastatin (SIM), metformin (MET) and their combination (SIM+MET). The absorbance was performed at 480 nm.

### Combination SIM and MET ameliorates metabolic aberrations of 22Rv1, C4-2B ENZU, C4-2B parental cells

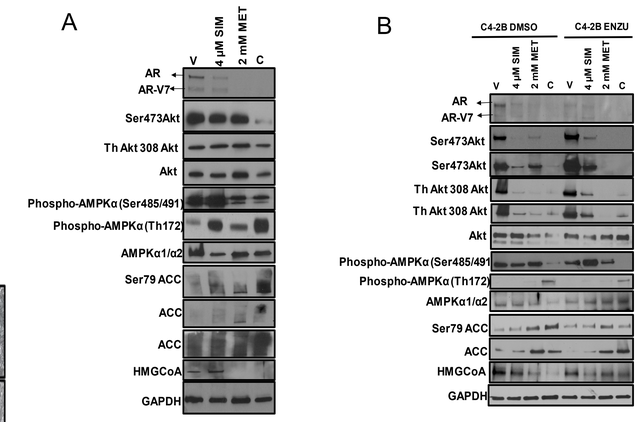


**Figure 7:** Western blot analysis of indicated antibodies in (A) 22Rv1 (B) C4-2B-ENZU resistant and their parental counterparts treatment with simvastatin (SIM), metformin (MET) and their combination (SIM+MET). GAPDH serves as loading control.

### Combination SIM and MET causes cell cycle arrest in the G0-G1 phase in CRPC cells

| 22Rv1 cells | Control | SIM (4μM) | MET (2mM) | SIM+MET |
|---|---|---|---|---|
| G0-G1 phase | 44.95% | 70.90% | 47.61% | 75.64% |
| G2-M | 18.81% | 11.05% | 21.42% | 8.80% |
| S | 36.42% | 18.05% | 30.97% | 15.56% |

**Figure 8:** Combination simvastatin and metformin treatment induces significant, sustained G1-phase cell cycle arrest in 22Rv1 cells. 22Rv1 cells were treated with 4 μM simvastatin (SIM) and 2 mM metformin (MET) for 72 h followed by staining with propidium iodide and analysis by flow cytometry..

### Conclusion

- Our results suggest that the combination SIM and MET may be an effective regimen for treatment ENZU-resistant prostate cancer cells.
- Our studies have identified an effective, inexpensive alternate chemotherapy with an excellent safety record that would greatly benefit quality-of-life of patients with biochemical recurrence as a result of ENZU resistance perhaps metastasizing to bone.

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