Role of DNA Methyltransferases and EZH2 in Enzalutamide Resistant Prostate Cancer

Brandon E. Burow[1,5] , Santosh Lamichhane[1,4,5], Sweaty Koul[1,3,5] and Hari K Koul[1,2,3,4,5]

Departments of [1]Interdisciplinary Oncology, [2]Biochemistry & Molecular Biology, [3]Urology, LSUHSC-New Orleans; [4]Southeast Louisiana Veterans Health Care System, New Orleans – LA; and [5]LSU-LCMC Cancer Center, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA, USA

Introduction

Prostate cancer is the most prevalent malignancy among men in the United States, resulting in over 35,000 deaths annually (1). It originates primarily from the luminal epithelial cells of the prostate gland, which arise from basal cells during prostate development and differentiation and are characterized by the expression of the androgen receptor (AR) and other specific markers. While androgen signaling is essential for the normal function and survival of luminal cells, it is also pivotal in the development and progression of prostate cancer (6).

Androgen deprivation therapy (ADT) has long been a cornerstone in the treatment of advanced prostate cancer. By reducing levels of androgens, ADT aims to inhibit androgen receptor signaling and therefore reduce the growth and proliferation of prostate cancer cells (5). However, despite the initial efficacy of ADT, most patients eventually develop resistance, leading to the emergence of castration-resistant prostate cancer (CRPC). Enzalutamide (ENZA), a next-generation AR inhibitor, has been approved by the FDA for treating metastatic CRPC (mCRPC). ENZA works by inhibiting the androgen receptor signaling pathway. Clinical trials have demonstrated that ENZA extends overall survival and delays disease progression. However, the majority of patients who initially respond to ENZA therapy eventually develop resistance, known as Enzalutamide Resistance (ENZ-R) (3).

The development of ENZ-R is marked by adaptive cellular mechanisms, including cellular plasticity and the emergence of neuroendocrine prostate cancer (CRPC-NEPC) and double-negative prostate cancer (CRPC-DNPC) phenotypes. While genomic alterations have been extensively investigated in ENZ-R, the role of epigenetic mechanisms remains less understood. Recent studies, including those from our laboratory, have pointed to increased expression and activity of DNA methyltransferases (DNMTs) such as DNMT1, DNMT3a, and DNMT3b, and the polycomb repressive complex 2 (PRC2) component EZH2 during prostate cancer progression (2,4). However, the direct role of DNMTs in ENZ-R has not been thoroughly evaluated.

In this study, we explore the potential of DNMT and EZH2 inhibitors to sensitize prostate cancer cells to enzalutamide. 5-Aza-2'-deoxycytidine (5-AZA-dC) is a broad-range DNMT inhibitor that incorporates into DNA and covalently traps DNMTs, leading to DNA hypomethylation and reactivation of silenced genes. GSK-126 is a selective EZH2 inhibitor that impedes the methyltransferase activity of EZH2, reducing the tri-methylation of histone H3 on lysine 27 (H3K27me3), which is associated with gene repression. Our results show that targeting these epigenetic regulators, may help overcome ENZA resistance in Prostate Cancer.

Methods

.

1. **Cell Culture**: LNCaP and LNCaP-ENZ-R cell lines were cultured under standard conditions with the LNCaP-ENZ-R receiving consistent treatment with 5uM ENZ.
2. **Western Blot Analysis**: Protein expression levels of DNMT and other prostate cancer markers were determined using Western blotting techniques.
3. **PCR Analysis**: DNMT gene expression was quantified through polymerase chain reaction (PCR) assays.
4. **Cell Growth and Proliferation**: The IncuCyte live-cell analysis system was utilized to monitor and measure cell growth and proliferation rates.
5. **Colony Formation Assay**: The ability of single cells to grow and form colonies was assessed using colony formation assays.

Fig 1. Cell Morphology of LNCaP and LNCaP-ENZ-R Cells

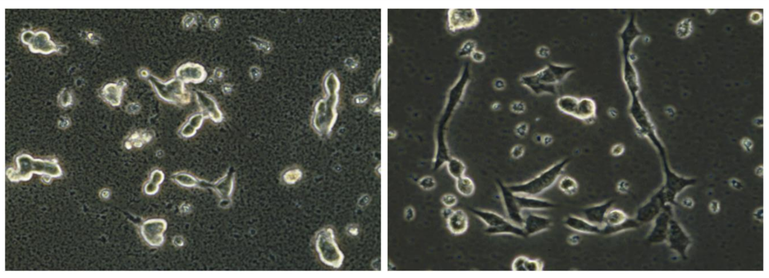


Fig 1: LNCaP cells show cuboidal epithelial morphology, while ENZR-LNCaP cells show Neuro-endocrine morphology

Fig 2. Enzalutamide Resistance is associated with increased DNMT Expression

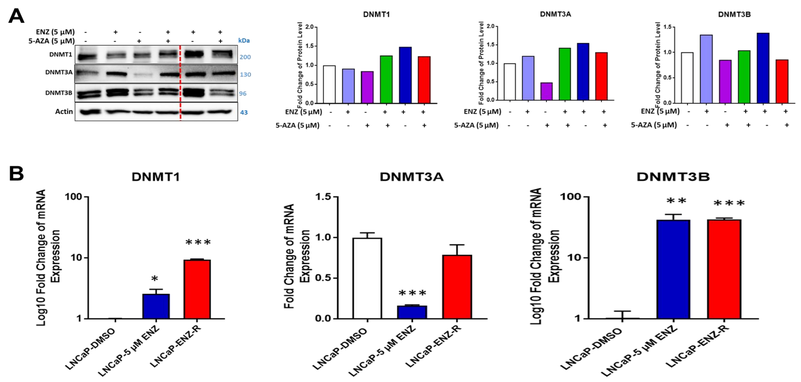


Fig Effects of Enzalutamide treatment on DNMT levels: Protein levels of DNMT1, DNMT3A, and DNMT3B were assessed in LNCaP and LNCaP-ENZ-R cell lines treated with 5 μM ENZ, 5 μM 5-AZA, or a combination of both. Actin was used as a loading control. **Fig 2a.** Quantification of Protein Expression: Bar graphs representing the quantification of DNMT1, DNMT3A, and DNMT3B protein levels from the Western blot data. The graphs show relative protein expression normalized to actin in LNCaP and LNCaP-ENZ-R cells under different treatment conditions; **2b:** Gene Expression Analysis: PCR quantification of DNMT1, DNMT3A, and DNMT3B mRNA levels in LNCaP and LNCaP-ENZ-R cell lines under the same treatment conditions as in (A). The results are presented as log10 fold changes relative to untreated controls. Statistical significance is indicated (*p < 0.05, ** p < 0.01, *** p < 0.001).

Fig 3. Enz-Resistant PCa cells are sensitive to DNMT inhibition, and Inhibition of DNMT sensitizes PCa cells to ENZ

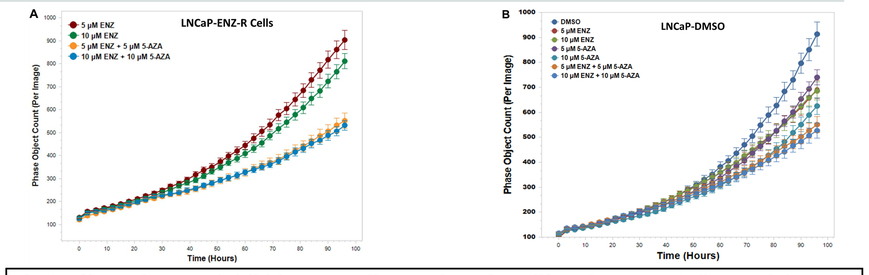


Fig.3 Effect of DNMT inhibition on call growth and proliferation: 3a: Cell Growth in LNCaP-ENZ-R Cells treated with varying concentrations of ENZ (5 μM and 10 μM), 5-AZA (5 μM and 10 μM), and their combinations were monitored 96 hours using the IncuCyte live-cell analysis system; **3b:** The growth and proliferation of LNCaP cells treated with varying concentrations of ENZ (5 μM and 10 μM), 5-AZA (5 μM and 10 μM), and their combinations were monitored 96 hours using the IncuCyte live-cell analysis system. Cell Density: 3k cells per well, 200uL media

Fig 4. Inhibition of DNMT's decreased clonogenic activity of ENZ-Resistant PCa cells

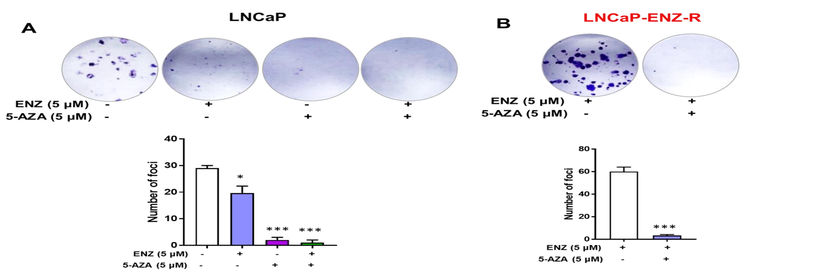


Fig 4 Effects of DNMT inhibition on Colony Formation : **A**: Representative images of colonies formed by LNCaP cells following treatment with 5 μM ENZ, 5 μM 5-AZA, and their combination. Colonies were stained and quantified after a incubation period. The bar graph below depicts the number of colonies formed under each treatment condition; **B:** Representative images of colonies formed by LNCaP-ENZ-R cells following treatment with 5 μM ENZ alone or in combination with 5 μM 5-AZA . The bar graph below indicates a significant reduction in colony formation in LNCaP-ENZ-R cells treated with the combination of ENZ and 5-AZA. Statistical significance is indicated (* p < 0.05, *** p < 0.001).

Fig 5. Enzalutamide Resistance is associated with increased EZH2 Expression.

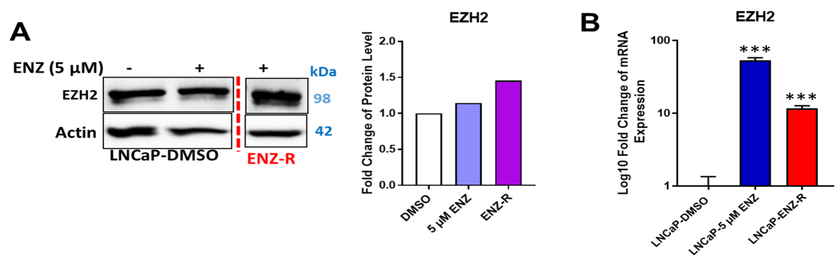


Fig 5 Effect of Enzalutamide on EZH2 Levels: A) Protein expression levels of EZH2 were assessed in LNCaP and LNCaP-ENZ-R cell lines treated with 5 μM ENZA. Actin was used as a loading control, and the quantification of EZH2 protein levels from the Western blot data; **B:** EZH2 mRNA levels in LNCaP and LNCaP-ENZ-R cell lines were measured via Realtime PCR. The results are presented as log10 fold changes relative to untreated controls. (*** p < 0.001).

Fig 6. PCa cells are sensitive to the inhibition of DNMT and EZH2: Dual inhibition of DNMT's and EZH2 decreased growth and proliferation of ENZ-R-PCa cells

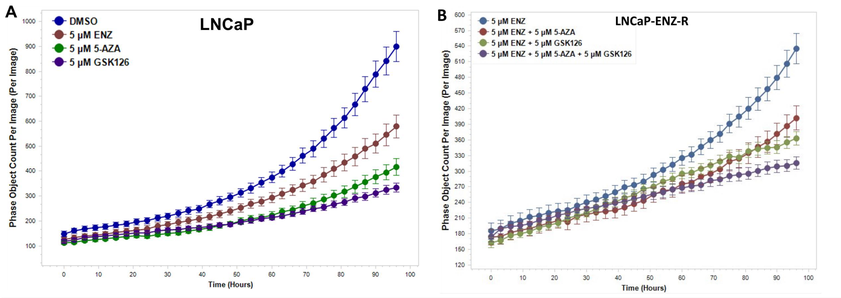
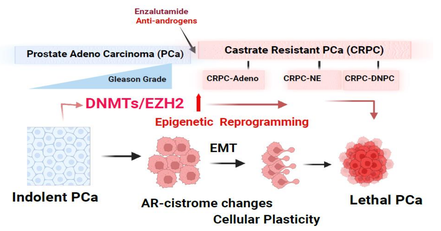


Fig 5a. Cell Growth in LNCaP Cells: The proliferation of LNCaP cells treated with 5 μM ENZ, 5 μM 5-AZA, 5 μM GSK126, and their combinations were monitored over 96hours using the IncuCyte live-cell analysis system. **Fig 5b.** Cell Growth in LNCaP-ENZ-R Cells: The proliferation of LNCaP-ENZ-R cells under the same treatment conditions as in (A) was monitored similarly.

Conclusions:

Our data demonstrated that the DNA/Histone methylation pathways are deregulated in ENZ-R Prostate cancer cell lines, and that targeting DNA methyl-transferases and EZH2 sensitized the prostate cancer cells enzalutamide (current treatment).



These studies suggest DMNT and EZH2 activities as a potential therapeutic vulnerability that can be exploited for limiting cellular plasticity, tumor progression, and therapy resistance in prostate cancer. Because DNMT and EZH2 inhibitors are currently approved for other malignancies, addition of these inhibitors to current treatment regiments could be readily explored in PCa.

References

1. American Cancer Society. (2024). *About prostate cancer*. Retrieved from https://www.cancer.org/cancer/types/prostate-cancer.html
2. Cheng, H., Tang, S., Lian, X., Meng, H., Gu, X., Jiang, J., & Li, X. (2021). The differential antitumor activity of 5-Aza-2'-deoxycytidine in prostate cancer DU145, 22RV1, and LNCaP cells. *Journal of Cancer*, *12*(18), 5593.
3. Linder, S., van der Poel, H. G., Bergman, A. M., Zwart, W., & Prekovic, S. (2019). Enzalutamide therapy for advanced prostate cancer: efficacy, resistance and beyond. *Endocrine-related cancer*, *26*(1), R31-R52.
4. Shankar, E., Franco, D., Iqbal, O., Moreton, S., Kanwal, R., & Gupta, S. (2020). Dual targeting of EZH2 and androgen receptor as a novel therapy for castration-resistant prostate cancer. *Toxicology and Applied Pharmacology*, *404*, 115200.
5. Sharifi, N., Gulley, J. L., & Dahut, W. L. (2010). An update on androgen deprivation therapy for prostate cancer. *Endocrine-related cancer*, *17*(4), R305-R315.
6. Zhang D, Zhao S, Li X, Kirk JS, Tang DG. Prostate Luminal Progenitor Cells in Development and Cancer. Trends Cancer. 2018 Nov;4(11):769-783. doi: 10.1016/j.trecan.2018.09.003. Epub 2018 Oct 1. PMID: 30352679; PMCID: PMC6212301.

Acknowledgements: These studies were funded in part by NIH/NCI-R01CA242839 (HK), and VA Merit Award: I01BX005351(HK), and unrestricted funds from the LSU-LCMC Cancer Center, School of Medicine -LSUHSC, New Orleans (HK) and LCRC/LSU summer research program (SUCRE).