Combinatory inhibition of Aurora Kinases and EZH2 leads to a synergistic antitumor effect in TNBC

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Introduction

Triple-negative breast Cancer (TNBC) accounts for around 15-20% of all breast cancers and is associated with poor long-term outcomes. It remains the most challenging subtype to treat because of its aggressive phenotype and limited treatment options[1]. Aurora Kinases are overexpressed in multiple types of solid tumors, including breast cancer[2,3]. Our preliminary studies discovered that AURKB expression disproportionally increased in African American TNBC patients more than in European American TNBC patients. Aurora kinase inhibitors, such as alisertib (MLN8237), have demonstrated early signals of efficacy with a manageable safety profile for the treatment of patients with locally advanced or metastatic breast cancer (NCT02860000). However, they did not show significant efficacy in TNBC unless combined with chemotherapy. EZH2, a histone methyltransferase and catalytic subunit of Polycomb Repressive Complex 2 (PRC2), has a broad role in cell stemness, and its overexpression promotes the development of many cancers, including TNBC. Multiple independent studies have identified EZH2 as an attractive drug target in TNBC. Several EZH2 inhibitors, which inhibit the methyltransferase activity of EZH2, have been approved for the treatment of sarcoma and follicular lymphoma in clinical settings. However, EZH2 inhibitors are ineffective at inhibiting TNBC cell growth.

Methods

1. Western Blot[2]
- Cells were lysed in IP lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentration was determined using the BCA assay. Equal amounts of protein (60-70 µg) were separated on SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in TBS-T (0.1% Tween-20) for 30 minutes at room temperature, then incubated with primary antibodies overnight at 4 °C. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. Signals were detected using enhanced chemiluminescence (ECL) and imaged with a gel documentation system.

2. Co-Immunoprecipitation (co-IP)[2]
- Cells were lysed in IP lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing protease inhibitors. Lysates were then incubated with 2–5 µg of primary antibody overnight at 4 °C. Immune complexes were captured with protein A/G beads for 2 hours, washed extensively, and eluted by boiling in SDS sample buffer. Co-precipitated proteins were analyzed by Western blotting as described above.

Results

1

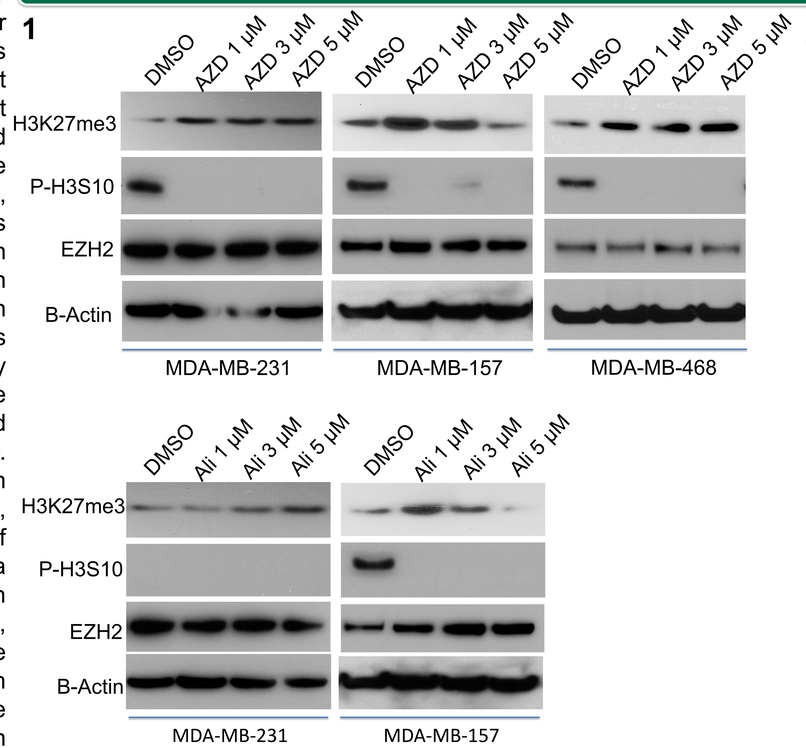


Figure 1: H3K36me2 expression is regulated by Aurora Kinases.
IB analysis of H3K27me3 and EZH2 expression in TNBC cells treated with AZD1152 or Alisertib in a dose course after pretreatment with Paclitaxel at 100 nM.

2A

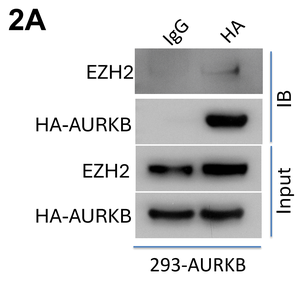


Figure 2: Co-immunoprecipitation reveals interaction between EZH2 and Aurora Kinases.
(**A**) Co-IP in 293-AURKB-HA cells using anti-HA antibody pulled down HA-AURKB.

3

EZH2 (human)	PNNssRPstPtINVL	S366-p
EZH2 iso2 (human)	PNNSSRPSTPTINVL	S371
EZH2 (mouse)	PNNssRPstPtISVL	S366-p
EZH2 (rat)	PNNSsRPStPtIsVL	S366

Figure 3. Aurora Kinases phosphorylate EZH2 at a potential serine 366 (S366) site.
Cell Signaling database alignment of EZH2 protein isoforms across species reveals a conserved serine residue at position 366 (S366) in the canonical human isoform. The sequence surrounding S366 (PNNssRPstPtINVL) is conserved in human, mouse, and rat EZH2, with the phosphorylation site (S366) indicated in Red. The site is at S371 position in the human isoform 2. This alignment supports the potential functional significance of S366 as a conserved phosphorylation site, possibly regulated by Aurora Kinases.

4

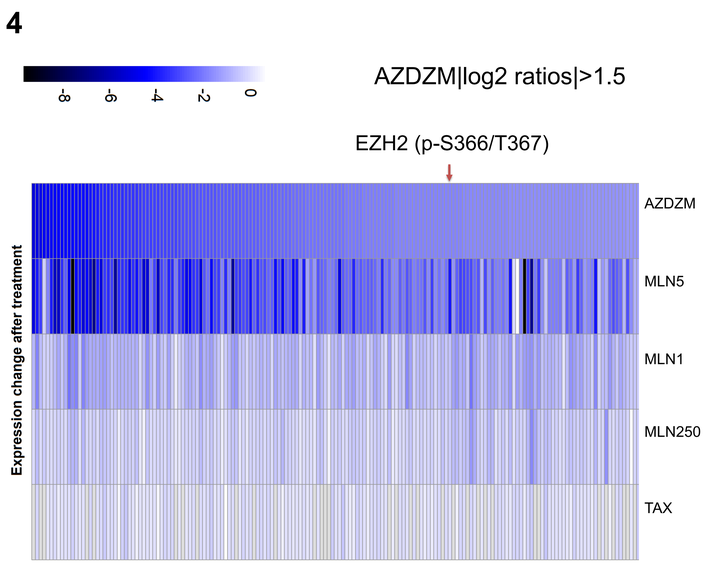
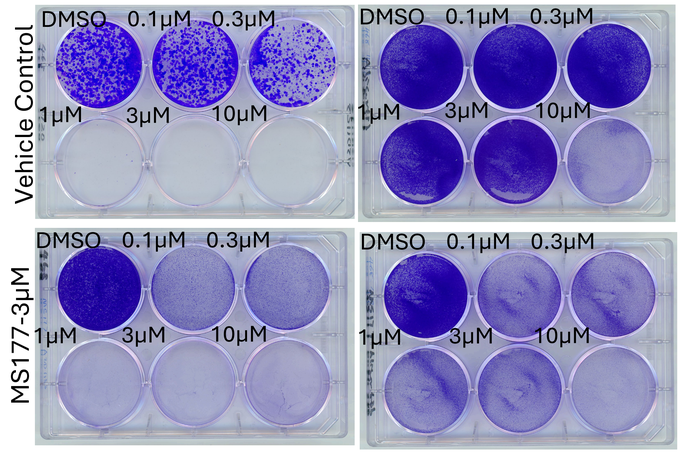


Figure 4. Aurora Kinases phosphorylate EZH2 at S366/T367 sites.
Heatmap illustration of AURKA and AURKB phosphorylation substrates identified by quantitative phosphoproteomics with ratios reduced in the MLN8054 or AZDZM-ZM447439 (5µM) or AZD1152 (1µM) conditions by 2.5-fold or more relative to uninhibited cells.

5A



5B

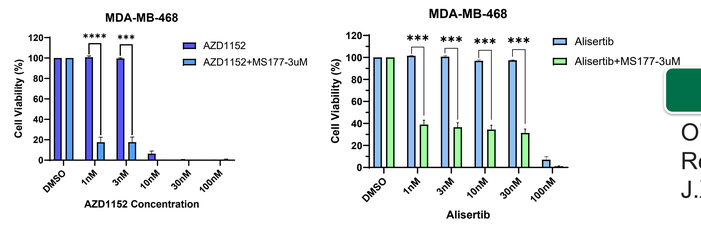


Fig. 4. Representative image of (**A.**) and quantification (**B.**) of Colony Formation assay analysis of indicated cells treated with AZD1152 or Alisertib in indicated concentration combined with MS177 (3µM). Unpaired t test with Welch' correction. ****, P≤0.0001; ***, P≤0.001, **, P≤0.01: *, P≤0.05.

Conclusions and Future Directions

- Aurora Kinases interact with and phosphorylate EZH2, and inhibition of Aurora Kinases upregulates the chromatin silencing marker H3K27me3.
- Combined inhibition of EZH2 and Aurora kinases shows a synergistic antitumor effect in TNBC.
- Phosphorylation of EZH2 by Aurora Kinase could retain EZH2 in the cytosol, leading to a functional shift from a PRC2 complex-dependent activity to a potential non-canonical EZH2 pro-metastatic function.
- Combinatory treatment leads to a synergistic effect on this panel of gene expression, which is critical for cell apoptosis, proliferation, and metabolism in TNBC cells.
- This non-canonical function highly depends on EZH2-mediated cancer cell apoptosis, proliferation, invasion, and metastasis, which differs from the well-known cell division function of Aurora Kinases, regulating chromosomal alignment, segregation, and cytokinesis during mitosis.

References

1. Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nat Rev Clin Oncol.* PMID: 27184417;
2. Xu J, Yu X, Martin TC, Bansal A, Cheung K, Lubin A, Stratikopoulos E, Cahuzac KM, Wang L, Xie L, Zhou R, Shen Y, Wu X, Yao S, Qiao R, Poulikakos PI, Chen X, Liu J, Jin J, Parsons R. AKT Degradation Selectively Inhibits the Growth of PI3K/PTEN Pathway-Mutant Cancers with Wild-Type KRAS and BRAF by Destabilizing Aurora Kinase B. *Cancer Discov.* PMID: 34301793;
3. Chung S, Sperier E, Graciano K, Hu X, Guo H, Xu J. Aurora kinases, emerging critical targets for cancer treatment and related new therapeutic strategies. *Bioorg Chem.* PMID: 40446708.

Acknowledgments

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