

# Effects of Rigosertib (RIGO) Alone or in Combination with Azacitidine or Vorinostat on Epigenetic Reprogramming of CD34+ Cells in the Myelodysplastic Syndrome

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## BACKGROUND

Azacitidine (AZA) is the standard of care for patients (pts) with higher-risk MDS, however, only 50% of pts respond and the majority will relapse within 2 years. All pts ultimately fail treatment due to primary or secondary resistance. RIGosertib (RIG) is a "ras mimetic" agent that binds to the Ras Binding Domain of RAF kinases and inhibits the RAS-RAF-MEK and the PI3K pathways. Initial results of an ongoing Phase I/II study with RIG combined with AZA, in pts with MDS demonstrated a response rate of: 76% overall; 62% in pts following hypomethylating agent (HMA) failure and 85% in HMA naïve pts (Navada *et al*, EHA, 2017).

## METHODS

**In vitro Study.** We investigated the in vitro effects of RIGO combined with AZA on two cell lines: AML (BW90), MDS (MDS-L) cells and pt bone marrow samples obtained prior and after 1 cycle of AZA and RIG. MDS-L and BW90 cells were initially primed in serum-free StemLinell (Sigma-Aldrich) media overnight and treated with AZA, RIGO, AZA/RIGO or RIGO/AZA for 48 hrs.

**Q-PCR assay.** Total RNA was extracted from AZA or RIGO treated MDS-L and BW-90 cells and pts BM samples, c-DNA was prepared and Q-PCR assays were performed using Sybre Green.

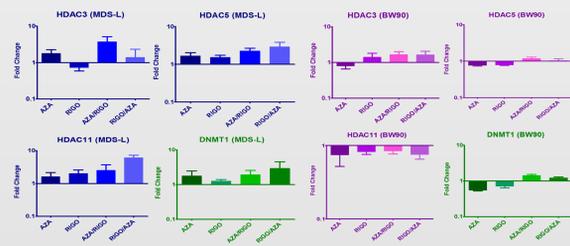
**Histone post-translational modifications assay.** To identify cell populations with high and low levels of active (H3K4me2, H3K9ac and H3K18ac) and repressive (H3K4me3, H3K27me3, H3K27me2) histone marks in CMA treated cells were stained with mAb according to the manufacturer's instructions (Cell Signaling Technology) and analyzed by using BD FACSCanto™ II Flow Cytometer.

**Western blot.** Whole-cell extracts were prepared from MDS-L and BW-90 cells after treatment with various drugs either alone or in combination for 48 hrs. Total cellular proteins were separated by SDS-PAGE and transferred by iBlot (Invitrogen). The Western-blot membranes were probed with mAbs against proteins from AKT, Cell cycle, Cdc25 signaling pathway and β-actin; Cell Signaling Technology) and developed using a chemiluminescence as per manufacturer's instructions.

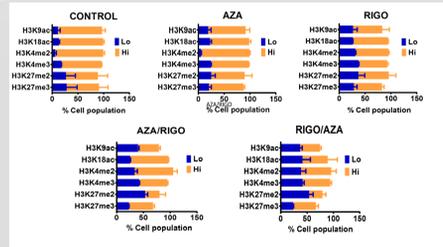
## REFERENCES

Navada *et al* Combination of Oral Rigosertib and Injectable Azacitidine in Patients with Myelodysplastic Syndromes (MDS): Results from a Phase II Study. Blood, December 2016 ASH abstract

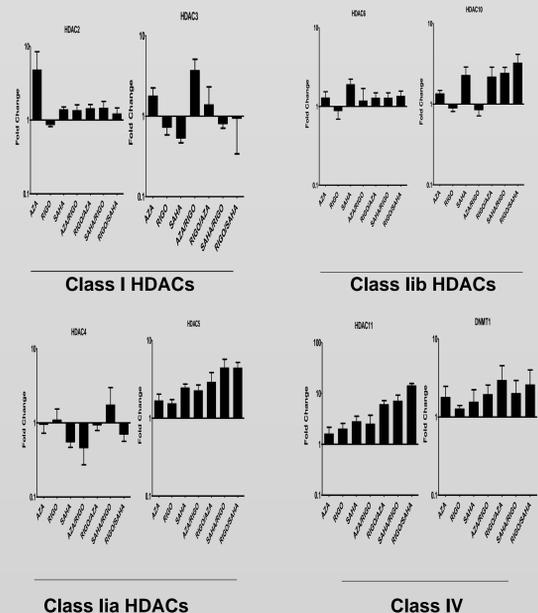
**RIGO modulates HDACs (class I, II and IV) and DNMT1 differentially in cell specific manner.** MDS-L and BW90 cells were treated with AZA, RIGO, AZA/RIGO or RIGO/AZA for 48 hrs and Q-PCR using SybrGreen was performed. Fold change in relative transcripts expression levels of HDACs and DNMT genes in MDS-L and BW90 are given below



**RIGO alone or in combination with AZA leads to different levels of histone methylation and acetylation in MDS-L.** Histone acetylation is considered as one of the marker of actively transcribed genes and in the presence of RIGO acetylation levels of the H3K9ac and H3K18ac were greatly increased as compared to AZA.



Flow cytometry reveals the existence of different levels (Lo-low and Hi-high) of histone H3 lysine-4, lysine-27 methylation (H3K4me2, H3K4me3, H3K27me2 and H3K4me3) and histone K3 lysine-18 and lysine-9 acetylation (H3K18ac and H3K9ac) following treatments with AZA, RIGO, AZA/RIGO and RIGO/AZA. On each row, the bar graph shows quantification of the percentage of MDS-L cells representing Lo and Hi distribution of various histone marks treated with AZA, RIGO, AZA/RIGO or RIGO/AZA for 48 hrs.



Fold change in expression levels of HDACs gene and DNMT1 in MDS-L (2A) Relative transcript levels of HDACs (Class I-HDAC, II and IV) and DNMT1 were calculated by SYBR Green Q-PCR.

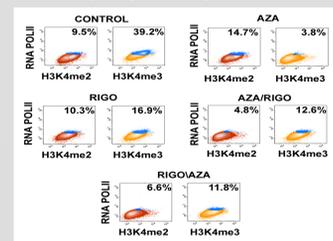
A relative mean normalized fold change in mRNA expression from each treatment conditions were normalized with the expression of these same genes in control cells. N=3-5

## RESULTS

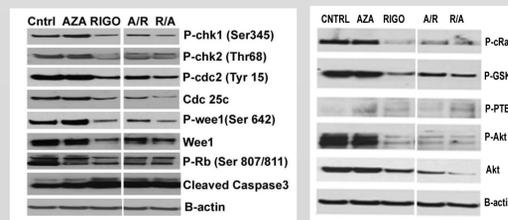
	CD34+	ALDH	Pluripotency Genes (SOX2; OCT4; NANOG; ZIC3)
Rigosertib	No increase	+++	+++
Azacitidine	3.8 x increase	Decrease	
Vorinostat	4 x increase	Decrease	
Rigosertib + Azacitidine	No increase	+++	+++
Rigosertib + Vorinostat	1.9x increase	Decrease	+++

Epigenetic reprogramming of pluripotency genes, expansion of primitive HSPC with down-regulation of the PI3K/AKT pathway and cell cycle checkpoint proteins may lead to enhanced hematopoietic function.

**Recruitment of RNA Polymerase II (Pol II) on altered histone marks.** Pol II is recruited on the promoters of the protein-coding genes. The alteration of H3K4me2 and H3K4me3 showed reduced Pol II occupancy as compared to control in MDS.



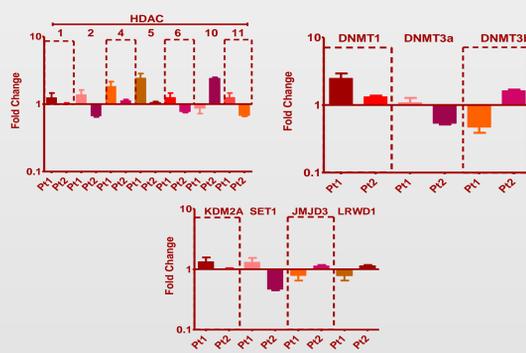
**Effect of RIGO alone or in combination with AZA on cell cycle check proteins, apoptosis and AKT cell signaling pathway.** Western blot analysis was performed on AKT signaling pathway proteins following 48 hrs of treatment with AZA, RIGO or AZA/RIGO RIGO/AZA. β-Actin expression as control.



Western blot analysis revealed that RIGO alone or in combination with AZA were more effective in downregulating AKT signaling and cell-cycle related proteins.

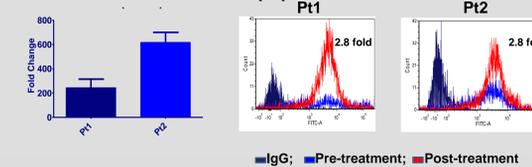
**Effects of RIGO on global histone post-translational modifications in MDS-L cells.** Flow cytometry reveals the existence of different levels (LO-low and HI-high) of histone H3 lysine-4, lysine-27 methylation (H3K4me2, H3K4me3, H3K27me2 and H3K4me3) and histone H3 lysine-9 and lysine-18 acetylation (H3K18ac and H3K9ac) following treatments with AZA, RIGO, AZA/RIGO or RIGO/AZA for 48hrs in MDS-L cells. On each row, the bar graph shows quantification of the percentage of MDS-L cells representing LO and HI distribution of various histone marks treated with CMAs either alone or in combination for 48 hrs. Two-way ANOVA P<0.0001.

**Levels of HDACs, DNMTs and chromatin remodelers transcripts in BM of MDS patients treated with 1 cycle of AZA and RIGO**

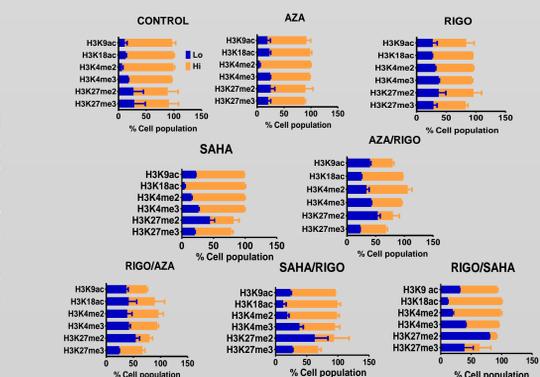
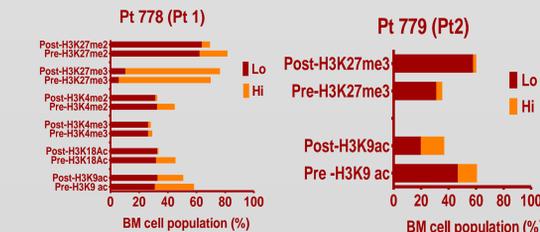


Relative transcript levels of HDACs (Class I, II and IV), DNMTs (1, 3a and 3b) and chromatin remodelers 1 cycle of RIGO and AZA treatment were calculated by SYBR Green Q-PCR.

**BM of MDS patients treated with 1 cycle of AZA and RIGO demonstrated enhanced transcripts levels of CD34 and apoptosis.**



**Histone post-translational modifications (PTMs) expression levels in MDS patients-BM prior and after 1 cycle of AZA and RIGO treatments.** Flow cytometry analysis revealed altered histone post translational modifications in both MDS patients.



## CONCLUSIONS

- The epigenetic events modulated by RIG in combination with either AZA or VOR led to:**
  - Global histone PTMs
- Differential Pol II association with active histone marks,
- Epigenetic reprogramming of pluripotency genes
- Expansion of primitive HSPC
- Downregulation of the PI3K/AKT pathway and cell cycle checkpoint proteins.
- Epigenetic effects of RIG on chromatin alterations lead to HSPC reprogramming.
- These epigenetic changes may reverse clinical epigenetic resistance and lead to enhances hematopoietic function and response in the clinical setting.
- These preclinical models suggest potential novel clinical strategies to improve outcomes for patients with higher-risk MDS.