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 III 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).

**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 AASH abstract

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**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 RIGO. MDS-L and BW90 cells were initially primed in serum-free StemLineII (Sigma-Aldrich) media overnight and treated with AZA, RIGO, AZA/RIGO or RIGO/IAZA for 48 hs.

**Q-PCR assay.** Total RNA was extracted from AZA or RIGO treated MDS-L and BW90 cells and pts BM samples, cDNA 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, H3K8ac and H3K18ac) and repressive (H3K4me3, H3K27me3, H3K27me2) histone marks in CMA treated cells were stained with mAib 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.

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**RESULTS**

**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/IAZA for 48 hrs and Q-PCR using SYBRGreen was performed. Fold change in relative transcript expression levels of HDACs and DNMT1 genes in MDS-L and BW90 are given below.

**Rigosertib 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 markers of actively transcribed genes and in the presence of RIGO acetylation percentage of MDS-L cells representing Lo and Hi distribution of H3K18ac and H3K9ac were greatly increased as compared to AZA.

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

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

**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.

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**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 AASH abstract