Rigosertib (RIGO) in combination with Azacitidine (AZA) modulates epigenetic effects and can overcome clinical resistance to hypomethylating agents (HMA) in Myelodysplastic Syndromes (MDS)

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**ABSTRACT**

MDS is a challenging disease to treat due to intricate complexities at the molecular, genetic and epigenetic levels. AZA is the standard of care for patients (pts) with MDS prior to allogeneic hematopoietic stem cell transplantation. In a single arm Phase I/II study of AZA combined with RIGO, an allostatic “ras mimetic” that binds to the Ras Binding Domain including ras, raf and PI3 Kinase pathways, in pts with MDS demonstrated an overall response rate of 77%; 84% in pts following HMA failure (Navada et al ASH 2015). We demonstrate that RIGO is a chromatin modifying agent (CMA) that epigenetically affects histone deacetylases (HDACs), DNA methyltransferases (DNMT) and histone post-translational modifications (PTMs) including acetylation and methylation in a cell specific manner. RIGO also modulates association of RNA polymerase II (Pol II) at active loci: on permissive histone marks (H3K4me3/H3K4me2) in MDS-L. In addition, RIGO downregulates more profoundly cell cycle related proteins, PI3/AKT pathway and upregulates cleaved caspase-3 in MDS-L cells. Similarly, sequential epigenetic therapy by 1 cycle of RIGO in AZA resistant MDS pts induces epigenetic effects on histone methylation and acetylation expression levels, HDACs, DNMTs and chromatin remodelers (KDM2A, SET1, JMJD3 and UHRF1) that resulted into altered chromatin remodelling leading to enhanced apoptosis.

**RESULTS**

1. RIGO altered HDACs (class I, II and IV) and DNMT1 expression differentially in a cell specific manner. MDS-L and BW90 cells were initially primed in serum-free StemLine II (Sigma-Aldrich) media over-night and treated with an optimal concentration of each AZA, RIGO, AZA/RIGO or RIGO/AZA for 48 hrs.

   Q-PCR assay. Total RNA was extracted from MDS-L and BW-90 at the end of treatment and pts BM. cDNA was prepared and Q-PCR assays were performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) in Applied Biosystems Thermal Cycler. GAPDH, RPL0 and RPL13A were used as internal housekeeping genes.

   Histone post-translational modifications and RNA polymerase (Pol II) assay. To identify cell populations with high (Hi) and low (Lo) levels of active (H3K4me2, H3K9ac and H3K18ac) and repressive (H3K4me3, H3K27me3, H3K27me2) histone marks in CMA treated and untreated control (control) cells were stained with monoclonal antibodies (mAbs) according to the manufacturer’s instructions (Cell Signaling Technology). Cells were analyzed by BD FACSCanto™ II Flow Cytometer. Double staining using RNA PolymeraseII (Pol II)Abcam) were simultaneously performed in combination with H3K4me2/3 mAb as per manufacturer’s instructions.

   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 isolated by SDS-PAGE and transferred by Blot (Invitrogen). The Western-blot membranes were probed with mAbs/Ab’s against proteins from PI3/AKT pathway, Cell cycle check points and β-Actin (Cell Signaling Technology) and developed using a chemiluminescence as per manufacturer’s instructions.

   Flow cytometry reveals the existence of different levels (Lo and Hi) of histone H3 lysine (K4, K5-7 methylation (H3K4me2, H3K4me3, H3K27me3 and H3K27me2) and histone H3 lysine (K9 and K14 acetylation (H3K9ac and H3K14ac) following treatments with RIGO, AZA, RIGO/AZA or AZA/RIGA. 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 RIGO/AZA either alone or in combination for 48 hrs. The combination has greater impact than the single agents alone (n=3-5).

2. RIGO alone or in combination with AZA leads to different levels of histone methylation and acetylation in MDS-L. Histone acetylation is considered a marker of actively transcribed genes and in the presence of RIGO/AZA, acetylation levels of the H3K9ac, H3K18ac and methylation levels of H3K4me2/3 were reduced (increased levels of Lo) as compared to AZA alone and control (reduced levels of Lo), suggesting that different levels of expression and sets of histone post-translational modifications may influence gene activity.

3. RIGO in combination with AZA reduces association of RNA Polymerase II (Pol II) on histone active (or paused) histone marks. The quantification of Pol II shows decreased amounts in association with reduced H3K4me2/3 methylations (LoHi) as compared to control in MDS cells. Low levels of Pol II could be due to bivalent genes (at histone marks H3K4me3/H3K27me3) or binding of Polycomb group complexes.

4. Effect of RIGO alone or in combination with AZA on cell cycle checkpoint protein, PI3/AKT cell signaling pathway and apoptosis. RIGO alone or in combination with AZA was more effective in downregulating PI3/AKT signaling pathway and cell-cycle related proteins compared to AZA and control. The combination led to growth inhibition, cell cycle dysfunction and apoptosis in MDS-L cells. One of the representative of 3-5 independent experiments.

5. Histone post-translational modifications (PTMs) expression levels in MDS patients-BM prior and after 1 cycle of RIGO and AZA treatments. Epigenetic deregulation plays an important role in pathogenesis of MDS. Flow cytometric analysis revealed epigenetic alterations in histone post-translational modifications at the both MDS pts either pre- or post-treatment. The alteration of cellular states (e.g., differentiation, malignant transformation or apoptosis of BM cells) may be accompanied by altered PTM hallmarks.

6. Altered levels of HDACs, DNMTs and chromatin remodelers transcripts in BM of MDS patients treated with 1 cycle of RIGO and AZA. The chromatin remodelers work as ‘writers’ or ‘erasers’. They assist in the rearrangement of chromatin either from silenced state to a transcriptionally accessible state or vice versa. The differential transcripts levels of HDACs, DNMTs and chromatin remodelers are likely due to variable epigenetic effects of RIGO and AZA on chromatin remodeling and related gene activity in pts.

[Graphical representations of Q-PCR, Western blot, Flow cytometry, and histone modifications are shown here.]

**CONCLUSION**

1. RIGO is an epigenetic modifying agent and it exerts cell specific responses by modulating the HDACs and DNMT.

2. RIGO governed epigenetic effects on histone post-translational modification expression levels (Lo and Hi) in MDS-L cells.

3. RIGO in combination with AZA showed decreased association of RNA polymerase II (Pol II) on the active promotors. This could be due either to lower levels of expression of H3K4me2/3 or gene bound to Polycomb repressive complexes on promotors.

4. RIGO alone or in combination with AZA downregulated the PI3/AKT pathway and reduced cell cycle check point protein levels and demonstrated an increase in apoptoses in MDS-L cells.

5. MDS patients treated with RIGO combined with AZA demonstrated alterations in HDACs, DNMTs and chromatin remodeler expression levels, histones post-translational modifications leading to enhanced apoptoses in MDS-L cells. The effect of RIGO alone or combined with AZA are mediated through epigenetic events.

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