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 higher-risk disease, however, all pts ultimately fail treatment due to primary or secondary resistance. Overcoming AZA resistance is a clinical imperative. Initial results of a Phase I/II study of AZA combined with RIGO, an allosteric "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%; 64% 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 (DNMTs) 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, PI3Kinase/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 demonstrates induced epigenetic effects on histone methylation and acetylation expression levels, HDACs, DNMTs and chromatin remodelers (KDM2A, SET1, JMJD3 and LRWD1) that resulted into altered chromatin remodeling leading to enhanced apoptosis.

METHOD

In vitro culture. We investigated in vitro effects of AZA, RIGO, AZA/RIGO and RIGO/AZA on two cell lines: AML (BW90), MDS (MDS-L) cells along with MDS pts. bone marrow samples obtained prior and after 1 cycle of RIGO and AZA. MDS-L and BW90 cells were initially primed in serum-free StemLine II (Sigma-Aldrich) media overnight and treated with an optimal concentration of each AZA, RIGO, AZA/RIGO or RIGO/AZA for 48 hrs.

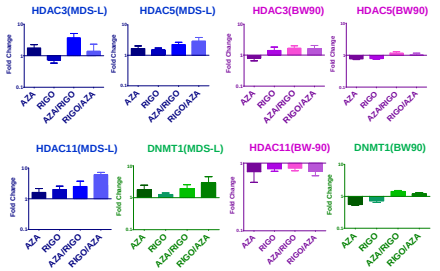
Q-PCR assay. Total RNA was extracted from MDS-L and BW-90 at the end of the treatment and pts BM sample. c-DNA was prepared and Q-PCR assays were performed using Sybre Green in Applied Biosystems Thermal Cycler. GAPDH, RPL0 and RPL13A were used as internal housekeeping genes.

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) cells were stained with monoclonal antibodies (mAb) according to the manufacturer's instructions (Cell Signaling Technology). Cells were analyzed by using BD FACSCanto™ II Flow Cytometer. Double staining using RNA Polymeraseil (Pol II)(Abcam) were simultaneously performed in combination with H3K4me2/3 mAb as per manufacturer's instructions.

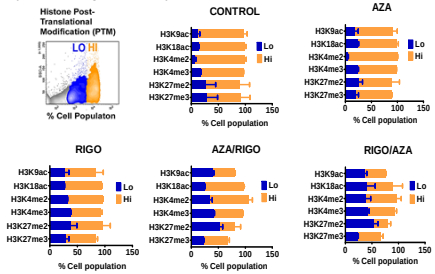
Western blot. Whole-cell extracts were prepared from MDS-L and BW-90 cells after treatment with various drugs either alone or in combination for 48 hrs. Total cellular proteins were separated by SDS-PAGE and transferred by iBlot (Invitrogen). The Western-blot membranes were probed with mAbs/pAbs against 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.

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 treated with AZA, RIGO, AZA/RIGO or RIGO/AZA for 48 hr and Q-PCR was performed. The histogram represents fold change in relative transcripts expression levels of HDACs and DNMT1 genes in MDS-L and BW90 cells. Histone hypoacetylation induced by HDAC activity is associated with gene silencing. Different levels of HDACs and DNMT1 suggest dynamic alterations in the cellular acetylome/DNA methylome and related chromatin structure on physiologically relevant pathways. Mean±SE (n=3-4)

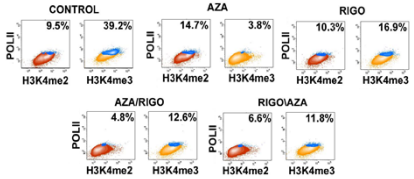


2. RIGO alone or in combination with AZA leads to different levels of histone methylation and acetylation in MDS-L. Histone acetylation is considered a marker of actively transcribed genes and in the presence of RIGO/AZA, acetylation levels of the H3K9ac, H3K18ac and methylation levels of H3K4me2/3 were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

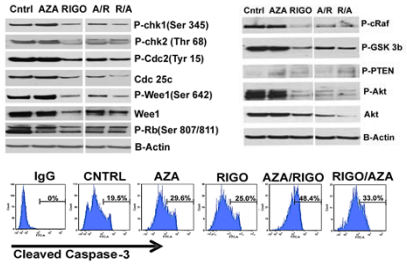


Flow cytometry reveals the existence of different levels (Lo-low and Hi-high) of histone H3 lysine (K)-4, (K)- 27 methylation (H3K4me2, H3K4me3, H3K27me2 and H3K4me3) and histone H3 lysine (K)-9 and K-18 acetylation (H3K18ac and H3K9ac) following treatments with AZA, RIGO, AZA/RIGO or RIGO/AZA. On each row, the bar graph shows quantification of the percentage of MDS-L cells representing Lo and Hi distribution of various histone marks treated with RIGO/AZA either alone or in combination for 48 hrs. The combination has greater impact than the single agents alone (n=3-5)

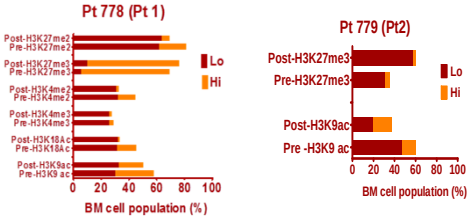
3. RIGO in combination with AZA reduces association of RNA Polymerase II (Pol II) on histone active (or paused) histone marks. The quantification of Pol II shows decreased amounts in association with reduced H3K4me2/3 methylations (Lo/Hi) as compared to control in MDS cells. Low levels of Pol II could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



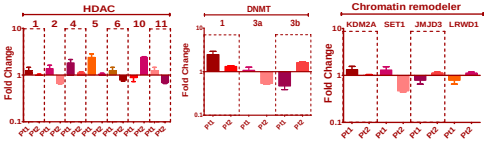
4. Effect of RIGO alone or in combination with AZA on cell cycle check point proteins, PI3/AKT cell signaling pathway and apoptosis. RIGO alone or in combination with AZA was more effective in downregulating PI3/AKT signaling pathway and cell-cycle related proteins compared to AZA and control. The combination 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 in 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.

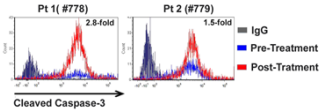


6. Altered levels of HDACs, DNMTs and chromatin remodelers transcripts in BM of MDS patients treated with 1 cycle of RIGO and AZA. The chromatin remodelers work as 'writers' or 'erasers'. They assist in the rearrangement of chromatin either from condensed state to a transcriptionally accessible state 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.



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

7. Apoptosis induced by 1 cycle of RIGO and AZA treatment in BM of MDS patients. Flow cytometric analysis revealed an increase in the apoptotic relative protein cleaved caspase-3 after 1 cycle of RIGO combined with AZA by 2.8 fold and 1.5-fold in MDS-L pts 1(#778) and 2 (#779), respectively.



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 promoters. This could be due either to lower levels of expression of H3K4me2/3 or bivalent genes bound to Polycomb repressive complexes on promoters.
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 apoptosis 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 apoptosis. The data suggest that the effects of RIGO alone or combined with AZA are mediated through epigenetic events.

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