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602.MYELOID ONCOGENESIS: BASIC

Azacitidine Combined with Novel Flavonoid Derivative GL-V9 Demonstrated Synergistic Anti-Leukemia Effect in Acute Myeloid Leukemia By Targeting DDIT4/mTOR Signaling

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Background Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy characterized by the clonal expansion of malignant progenitor cells and impaired cell differentiation. GL-V9, a nature compound derived from wogonin, has shown superior anti-tumor potential by inhibiting cancer cell growth. Azacitidine (AZA), a DNA hypomethylation agent, has demonstrated therapeutic efficacy against AML. In this study, we investigated the anti-leukemia effect and potential mechanism of AZA combined with GL-V9 in AML cells, aiming to provide evidence for future clinical treatment.
Methods We employed Cell Counting Kit-8 (CCK-8) to assess cell viability, Annexin-V/PI staining followed by flow cytometry analysis to measure apoptosis. CalcuSyn analysis was used to evaluate the synergistic effect. RNA-seq was performed in U937 cells and differentially expressed genes (DEGs) were identified and subjected to KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis. RT-qPCR and Western Blot were used to examine gene expression.
Results Our results demonstrated significant cell proliferation arrest in U937 and MV4-11 cells treated with the combination of AZA and GL-V9, compared to the single drug (**Fig. 1A&B**). CalcuSyn analysis showed a strong synergistic effect for the combination (**Fig. 1A&B**). Similar results were also found in primary AML cells derived from 2 AML patients [patient 1 exhibited t(6;11) (q27;q23) chromosomal translocation, while Patient 2 presented with secondary AML, transformed from CMML (Chronic Myelomonocytic Leukemia) and featured *ASXL1* and *SRSF2* mutations] (**Fig. 1C&D**). Moreover, AZA plus GL-V9 induced a substantial increase in cell apoptosis in U937 and MV4-11 cells compared to the single drug (**Fig. 1E&F**). Consistently, the AZA+GL-V9 combination led to a notable increase in the protein level of Bax, BAD, BIM, and a decrease in BCL2 (**Fig. 1G**). These data indicated the combination of AZA with GL-V9 has synergistic anti-leukemia effects in AML. To understand the underlying mechanisms of this synergy, we performed RNA-seq analysis in U937 cells, identifying 1385 and 586 DEGs ($|\log_2 FC| \geq 2.0$, $P<0.05$) upon AZA or GL-V9 treatment, respectively (**Fig. 2A**). The mTOR signaling pathway exhibits prominent enrichment in the KEGG analysis of the overlapping DEGs between AZA and GL-V9 (**Fig. 2B**). *DDIT4*, a negative regulator of mTOR emerged as one of the top DEGs in response to both AZA and GL-V9 treatment. Importantly, the AZA+GL-V9 treatment exhibited a higher expression level of DDIT4 compared to either drug alone and suppressed the phosphorylation of mTOR (**Fig. 2C**). Additionally, the *DDIT4* expression level was significantly decreased in AML patients, which derived from a public database (TCGA-AML, GSE13159)] (**Fig. 2D**) and Zhongda Hospital (Nanjing, China) compared to the healthy controls (**Fig. 2E**). To be noticed, high *DDIT4* expression was associated with extended overall survival ($P=0.021$) (**Fig. 2F**) and relapse-free survival ($P=0.015$) (**Fig. 2G**), indicating *DDIT4* may act as a tumor suppressor in AML. These findings suggested that the combination may exert the anti-leukemia effect by targeting the DDIT4/mTOR signaling pathway, as summarized in **Fig. 2H**.
Conclusions Our study provides novel evidence of the synergistic effect on cell growth arrest and apoptosis treated with a new nature compound derived from wogonin combined with AZA in AML. Furthermore, we identified the mechanism underlying the synergy through targeting of DDIT4/mTOR signaling. These results offer preliminary support for the potential application of this combination therapy in treating AML patients.

Disclosures No relevant conflicts of interest to declare.

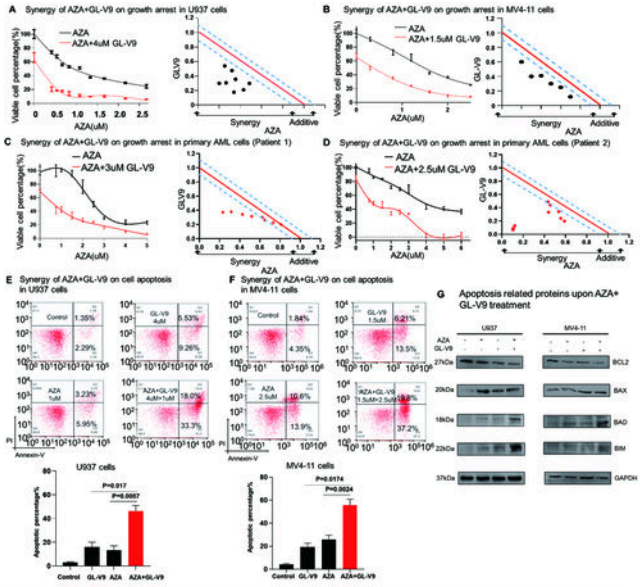

Fig 1 Synergistic effects of AZA + GL-V9 on cell proliferation arrest and apoptosis in AML cells. (A-D) Effects and synergistic analysis of AZA and the combination of AZA+GL-V9 on the proliferation of U937 (A), MV4-11(B), primary AML cells (patient 1) (C), and primary AML cells (patient 2) (D). (E-F) Effect of AZA, GL-V9, or combination on apoptosis of AML cells treated with AZA (1μM for U937 cells; 2.5μM for MV4-11) and/or GL-V9 (4μM for U937 cells; 1.5μM for MV4-11 cells) for 48 hours. (G) Effect of AZA, GL-V9, or combination on level of apoptosis-related proteins.

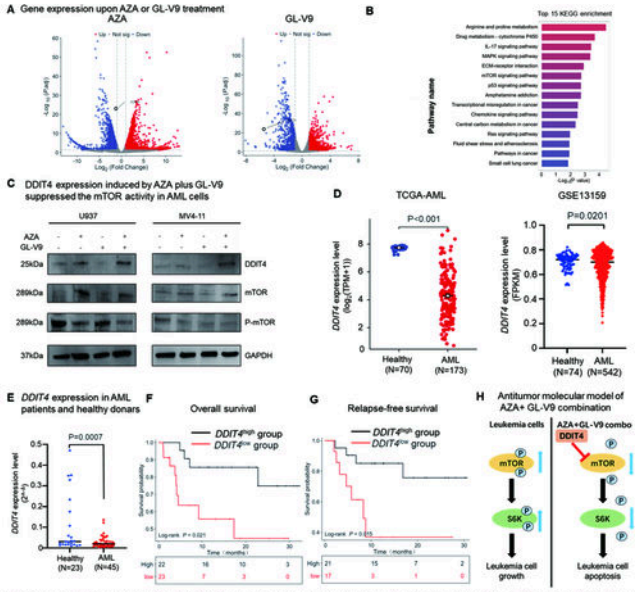

Fig 2 Synergistic molecular mechanism upon AZA plus GL-V9 treatment (A-B) Volcano plot of the differential expressed genes (DEGs) in U937 cells upon the treatment of AZA (left) or GL-V9 (right). (B) Enriched top 15 KEGG items for common altered genes upon AZA and GL-V9 treatment. (C) Protein levels of DDIT4, mTOR, and P-mTOR upon the AZA and/or GL-V9 treatment. (D-E) Expression levels of *DDIT4* in the AML patients from public database (D) and Zhongda Hospital (Nanjing, China) (E), compared to the healthy controls. (F-G) Overall survival (F) and relapse-free survival (G) of the high *DDIT4* expression group versus the low *DDIT4* expression group. (H) Model of anti-leukemic mechanism underling the AZA plus GL-V9 combination.

Figure 1

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