Peer Review File

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Reviewer A

In this manuscript, the authors reported that the BRD9 inhibitor I-BRD9 inhibited cell growth and induced cell death of AML cell lines. Besides, the authors analyzed the type of cell death induced by this inhibitor, demonstrating that cell death induced by I-BRD9 can be blocked by the caspase inhibitor Z-VAD-FMK. The present findings are interesting, yet there are several issues needed to be addressed before accepting this manuscript.

1. The authors show that I-BRD9 does not induce cell differentiation in AML cell lines. However, they only test this at 24 hours. It would be very useful measuring cell differentiation at 48 and 72 hours of treatment, as the other techniques such as cell viability or cell death.

As the reviewer suggested we have added more time points for the cell differentiation assay, here again, we showed that I-BRD9 does not induce differentiation of AML cells. We have replaced Fig.1d with new data.

2. Apoptosis markers (cleaved PARP, Caspase 3, etc) should be also analyzed after the combination of I-BRD9 and Z-VAD and included in Fig3B.

As the reviewer suggested we have done a new analysis combining I-BRD9 and Z-VAD for apoptosis markers. The new data clearly showed that Z-VAD treatment could reduce apoptosis markers induced by I-BRD9, including cleaved PRAR, Caspase3.

These have been updated in fig.3b.

3. Figure 2 and figure 3 might combine in only one figure which include all the experiments about cell death.

We really appreciated the reviewer's suggestion. However, due to the limitation of space and newly added data, we think separating Figure 2 and Figure 3 would fit better for presenting the data.

Reviewer B

In this manuscript, the authors treated two AML cells (MV4-11 and NB4) with a BRD9 inhibitor, I-BRD9. This treatment seems to reduce cell survival by inducing apoptosis. However, the current results are not sufficient to conclude that the effects were specifically caused by the inhibition of BRD9. Additional data are necessary to exclude the possible non-specific effects.

Other specific points:

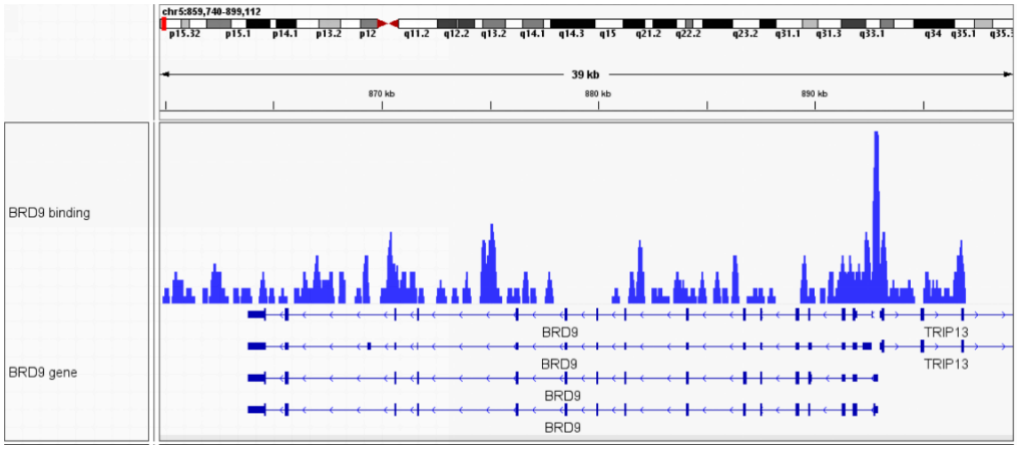
1. The authors only used one approach to inhibit BRD9. They need to validate the results using at least one other approach to inhibit BRD9, such as BRD9 RNAi.

We thank the reviewer for raising this question. Knockdown BRD9 by RNAi has been shown to inhibit multiple AML cell line growth, including the cell line we used in this study: NB4 [1]. It is also the basis of our study, and our goal is to extend the study to further evaluate whether targeting BRD9 by small molecule inhibitor could have a potential role in inhibiting AML cell growth.

2. In Fig. 4, why was BRD9 expression reduced upon I-BRD9 treatment if the

mechanism of I-BRD9 is to bind to BRD9?

We appreciate the reviewer noticed this phenomenon. We think BRD9 may regulate its own expression, so that inhibiting its activity resulted in decreased expression of BRD9. This is supported by the fact that BRD9 binds to its own gene in the genome, see figure below. We have included this discussion in the revised manuscript.



3. The authors need to show that other BRDs were not affected.

We have now included expression analysis of BRD1, BRD2, BRD3 after I-BRD9 treatment, and their expression level are not affected by I-BRD9 (Fig.3c). This has been updated in the revised manuscript.

4. In Fig.2, the authors measured the effect of Z-VAD-FMK on cell survival. To be consistent, they should also measure the effects on gene expression in Fig. 4 and Fig. 3b.

As the reviewer suggested, we have done a new analysis combining I-BRD9 and Z-VAD for apoptosis markers as well as gene expression in Fig. 3b and Fig. 4. The new data showed a rescue of Z-VAD on apoptotic markers induced by I-BRD9, but not on general gene expression. However, we do observe that Z-VAD treatment can reduce

CDKN1A and CDKN2B expression induced by I-BRD9. In addition, with more experiments been done, IRE3 also showed a significant change in I-BRD9 treated group compared to the control group, whereas in the previous manuscript it only showed a trend of induction in NB4 cells (Fig. 4). All the data have been updated in the revised manuscript.

5. Cell survival measurement should include more time points.

We have now including more time points for cell survival measurement, and replaced Fig.1a with new data.

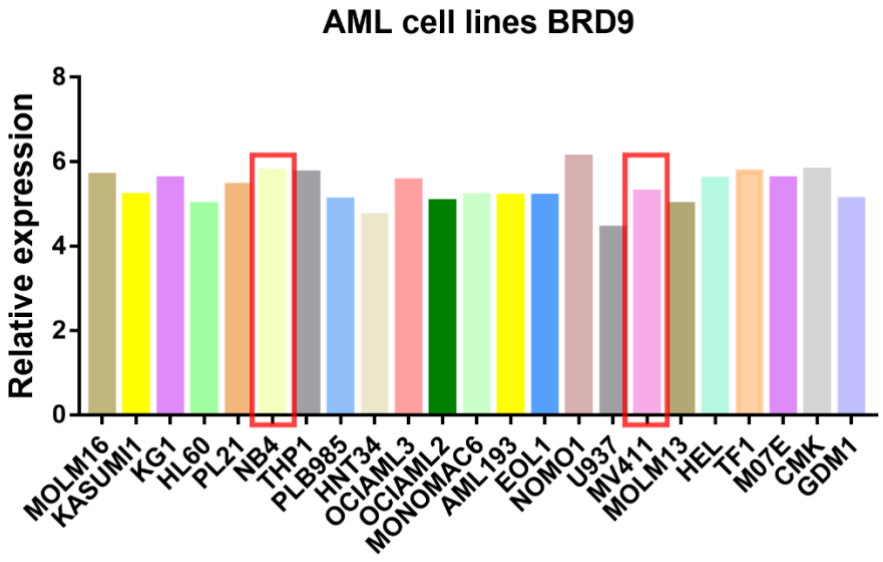
6. It is unclear why the authors decided to use MV4-11 and NB4 cells for this study. Was it because these cells over-express BRD9? If so, this needs to be mentioned in the manuscript.

Yes, NB4 has been shown to express a higher BRD9 level than normal CD34+ HSC cells[1]. According to our expression analysis is expressed as similar in MV4-11 cells as in NB4 cells (Fig.1a). We have now mentioned this in the manuscript.

7. To strengthen the conclusion, the authors should compare the current results with results from normal cells or from AML cells not over-expressing BRD9.

We thank the reviewer for raising this question. According to DepMap expression data (the Cancer Dependency Map, https://depmap.org), the majority of AML cell lines express a similar level of BRD9(see figure below), and it is difficult to find a normal human myeloid cell line. So we used a Diffuse large B-cell lymphoma (DLBCL) cell line, SU-DHL-4, which has a much lowered expression of BRD9 compared to NB4 and MV-4-11(Fig.1a). I-BRD9 has little or no growth inhibitor effect on SU-DHL-4 at the

same treatment condition as in NB4 and MV-4-11 cells (Fig.1a). This could suggest the on-target effect of I-BRD9 to inhibit the growth of BRD9 over-expression cells.



References

1. Del Gaudio, N., et al., *BRD9 binds cell type-specific chromatin regions regulating leukemic cell survival via STAT5 inhibition.* Cell Death Dis, 2019. **10**(5): p. 338.