3.5. Inhibition of ATM kinase activity induces apoptosis in AZA-resistant cell lines

To determine whether reversal of AZA resistance by KU55933 is due to the induction of apoptosis via the DNA damage response, we quantified the annexin V-positive cells in AZA-treated U937 and R-U937 cells (Fig. 8D). Annexin V-positive cells were significantly increased in AZA-treated U937 cells, whereas such an increase was not evident in AZA-treated R-U937 cells, as shown in Fig. 1C. Annexin V-positive cells were significantly increased in the presence of KU55933 in AZA-treated R-U937 cells. This indicates that the DNA damage response, via the activity of ATM kinase, is indeed involved in the AZA-resistant phenotype. In contrast, PARP inhibition by 1 μM olaparib did not cancel the AZA resistance in R-U937 cells. Taken together, these results show that the DNA damage response pathway involving ATM, rather than PARP, plays a key role in the AZA-resistant phenotypes.

4. Discussion

The demethylating agent AZA has received FDA approval for the treatment of MDS. However, the reason why a subset of patients is resistant to AZA remains unclear. In terms of clinical practice, there are two types of AZA resistance. The first type is primary treatment failure, namely, non-responders. The second type is acquired AZA resistance during the course of AZA-treatment. The molecular mechanism involved might be different between the two types of AZA resistance. In the current study, we demonstrated that the suppression of UCK2, which is involved in pyrimidine metabolism, was detected in the AZA-resistant cell lines, which is consistent with previous studies [20]. Several studies reported that the differences in AZA sensitivity among cell lines correlate with the differences in the expression levels of UCK2 [24,25]. Furthermore, recent studies demonstrated that genetic variations in UCK2 determine the sensitivity of cells to a cytidine analog [26,27]. Taken together, it is likely that the mechanism underlying acquired AZA resistance partially overlaps with that of intrinsic resistance. Further research using clinical samples will promote the understanding of the role of UCK2 expression, polymorphisms, and mutations in AZA resistance. We also identified POLR2B as another gene involved in AZA resistance. To the best of our knowledge, the role of POLR2B in AZA resistance has not been reported previously. The roles of POLR2B or the other subunits of POL II in AZA resistance should be clarified by further research.

Contrary to the detailed knowledge about the decreased incorporation of AZA into RNA in AZA-resistant cells, it is still unclear whether or not AZA is incorporated into DNA in AZA-resistant cells. DAC resistance and the DNA damage response in AZA-resistant cells used in the present study strongly suggest the incorporation of AZA into DNA in the resistant cells. The most important finding of this study is the involvement of ATM signaling in the AZA resistance of cells in which AZA has been incorporated into DNA. Indeed, the constitutive activation of ATM/BRCA1-dependent DNA repair and the inhibition of ATM/p53-dependent apoptosis prevented R-U937 cells from undergoing AZA-induced apoptosis. Although HL-60 cells do not possess the TP53 gene [28], the ATM inhibitors canceled the AZA-resistant phenotype of R-HL-60 cells, suggesting that AZA resistance involves the inhibition of p53-independent apoptosis. Previous studies have reported the constitutive phosphorylation of ATM in radiation-resistant cell lines and the radio-sensitization of these cells by ATM inhibitors [29,30], whereas the constitutive phosphorylation of ATM has never been reported in cells resistant to DNA-damaging agents. Since the AZA-resistant cell lines were exposed to clinical doses of AZA for several months for their establishment, the constitutive phosphorylation of ATM is likely to be linked to the mechanism

active in patients with acquired AZA resistance, whereas the role of the DNA damage response in AZA non-responders is still unclear.

It is widely thought that AZA and DAC exert their anti-tumor activities via the demethylation and reactivation of tumor suppressor genes [31]. If resistance to DNA demethylation is involved in AZA resistance, epigenetic silencing by the restoration of methylation in tumor suppressor genes is a candidate mechanism for gaining resistance to AZA. However, we did not find any increased methylation in the promoters of tumor suppressor genes in the AZA-resistant cell lines. Taken together with the results from the region-specific analysis of the promoters of UCK2 and POLR2B and global analysis of the DNA in AZA-resistant cells, our results indicate that restoration of DNA methylation is not involved in AZA resistance.

Unlike AZA, DAC is metabolized by DNA metabolism. Although AZA and DAC are considered to rarely induce cross-resistance [32], the AZA-resistant cell lines in this study were resistant to DAC, and this resistance was canceled by treatment with caffeine. Our results strongly suggest the possibility that DAC resistance in the AZA-resistant cell lines is dependent on the ATM/BRCA1 pathway. We observed that the viability of the AZA-resistant cell lines was higher than that of their parental cell lines upon MMC, ETP or CDDP treatment. This indicates that the constitutive activation of ATM may allow the cells to gain resistance to a wide range of DNA-damaging drugs. The development of cross-resistance to other drugs should be an important consideration when AZA is used clinically.

Treatment with the CTP synthase inhibitor 3-DU canceled the resistance of R-U937 and R-HL-60 cells to AZA, as well as to ATM inhibitors. Recently, Raynal et al. [33] demonstrated that 3-DU enhances the cytotoxic activity of DAC by enhancing the incorporation of DAC into DNA. 3-DU causes a reduction in the CTP pool, resulting in a reduction in the deoxy-CTP (dCTP) pool, which leads to enhanced incorporation of aza-dCTP into DNA due to less competition with dCTP. The metabolites of AZA might be incorporated into not only DNA but also RNA, but nevertheless, this mechanism is plausible for the observed restoration of AZA sensitivity by 3-DU in this study. Although the utility of CTP synthase inhibitors for leukemia therapy should be evaluated in a further study, our results indicate that targeting CTP synthase can be beneficial for the treatment of leukemia.

Another important issue that should be addressed is the possible role of DNMT3A in AZA resistance. DNMT3A methylates the non-methylated CpGs and plays important roles in the decision of cell fates of hematopoietic stem cells [34,35]. In the AZA-resistant cell lines with highly demethylated DNA, DNMT3A might function to re-methylate the CpGs and might be entrapped by AZA. Various studies showed that CD34+ cells from patients with myeloid malignancy demonstrate lower DNA methylation levels than the healthy CD34+ cells [36,37]. In the patients with MDS, the progenitor cells that differentiate from pathogenic CD34+ cells cannot proliferate or differentiate normally and die from apoptosis. The patients then suffer from severe ineffective hematopoiesis, which can be improved by treatment with AZA or DAC. On the other hand, a previous study showed the persistent proliferation of pathogenic CD34+ cells in patients treated with AZA [38]. Further characterization of our AZA-resistant cell lines will promote the understanding of the mechanism of action of demethylating agents in leukemia cells with hypomethylated DNA.

On the other hand, some limitations exist in our approach. Since R-U937 and R-HL-60 cells are not of clonal origin, they may be heterogeneous populations. Hence, we cannot determine whether multiple changes are needed to acquire resistance to AZA, or whether a single change can be responsible for AZA resistance. It is probable that the slow proliferation rate of AZA-resistant cell lines contributes to the AZA-resistant phenotype. However, we failed to

find any evidence, for example phase-specific prolongation of cell cycle, in the present study indicating that the slow proliferation rate of AZA-resistant cell lines is an active mechanism underlying AZA resistance. Finally, although we proposed a molecular mechanism underlying acquired AZA resistance, our results are not necessarily able to explain the AZA resistance mechanism in non-responders. Since approximately half of the patients with MDS show no response to AZA treatment, it is very important to determine whether or not the same mechanisms are involved in the acquired and primary resistance to AZA. These problems should be clarified by further studies both *in vitro* and *in vivo*.

In the present study, AZA-resistant cell lines were established and characterized at the molecular level. We propose that the molecular mechanism underlying resistance to AZA involves pyrimidine metabolism and sustained DNA damage response involving ATM as well as BRCA1. These findings provide new insights into the diagnostic and therapeutic strategies for the treatment of AZA-resistant MDS patients.

Competing interests

The authors declare no competing interests.

Acknowledgements

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