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RAPID COMMUNICATION

# BET inhibitors enhance the anti-cancer effect of etoposide by suppressing the MRN-ATM axis in the DNA damage response

Etoposide is widely used for cancer chemotherapy in the clinic. However, long-term etoposide treatment can lead to adverse effects or drug resistance. To improve the situation, we evaluated the therapeutic efficiency of etoposide combined with inhibitors of bromodomain and extra-terminal (BET) family proteins, which have recently emerged as novel anti-cancer targets due to their critical roles in cancer development. Firstly, we showed BRD4, one of the main targets of BET inhibitors, was involved in DNA damage response (DDR) via the homologous recombination (HR) repair pathway. Then we found BRD4 deficiency and inhibition reduced ataxia-telangiectasia mutated (ATM) recruitment and activation by down-regulating the expression of MRN complex (MRE11-RAD50-NBS1) proteins. Subsequently, our data showed that BET inhibitors could sensitize cancer cells to etoposide treatment both *in vitro* and *in vivo*. In summary, BRD4 inhibitors enhance the anti-cancer effect of etoposide by suppressing the MRN-ATM axis in DDR, thus indicating the important value of these inhibitors as candidates for combination with etoposide in chemotherapy.

Three BET inhibitors were employed in our study as followed.[1] JQ1 is the first studied BET inhibitor that binds competitively to the bromodomains of BET proteins. OTX-015 has shown efficacy in both hematological and solid cancers and has been applied in clinical trials. ARV-825 is a proteolysis targeting chimera (PROTAC)-based BRD4 degrader. Firstly, we showed that high BRD4 expression was associated with a low overall survival (OS) rate for multiple types of cancer patients, indicating that BRD4 is a potential therapeutic target for cancer (Fig. S1A). To study the roles of BRD4, we knocked down the expression of BRD4 in HeLa cells and treated cells with etoposide, the inducer of DNA damage, followed by releasing for the indicated period. Immunofluorescence assays and Western blot showed a marked increase in the phosphorylation of histone H2AX (γH2AX), a marker of DDR, and slower recovery in the BRD4-knockdown (KD) groups (Fig. 1A; Fig. S1B). Therefore, BRD4 deficiency led to the accumulation of DNA damage induced by etoposide, which was also verified by comet assays. Compared with control cells, the tail moments were significantly larger in BRD4-KD cells and cells treated with BET inhibitors following etoposide treatment and release for 0.5 h and 6 h, indicating more severe DNA damage and dramatically delayed DNA repair (Fig. 1B, C). These results suggested that BRD4 is involved in DNA damage repair caused by etoposide and BET inhibitors effectively restrain the function of BRD4 in DDR.

In DNA damage, double-strand breaks (DSBs) are more lethal to cells than single-strand breaks (SSBs). There are two major pathways of DSB repair, the HR pathway and the nonhomologous end-joining (NHEJ) pathway. By using DR-U2OS and EJ5-U2OS cells to analyze the efficiency of DNA repair mediated via HR and NHEJ respectively,[2,3] we found that BRD4 KD and JQ1 treatment significantly reduced HR efficiency, but not NHEJ efficiency (Fig. 1D, E), indicating that BRD4 is mainly associated with the HR pathway. KD of CtIP and XRCC4 were used as positive controls to verify the effectiveness of DR-U2OS and EJ5-U2OS cells respectively (Fig. S1C).

To elucidate the mechanism underlying the function of BRD4 in DDR, we investigated the role of BRD4 in regulating the expression of DDR proteins, since BRD4 is a transcriptional co-activator. In BRD4 knockout (KO) cells, the protein levels of MRE11, RAD50, and NBS1, factors in the HR pathway, were significantly lower than those in the BRD4-

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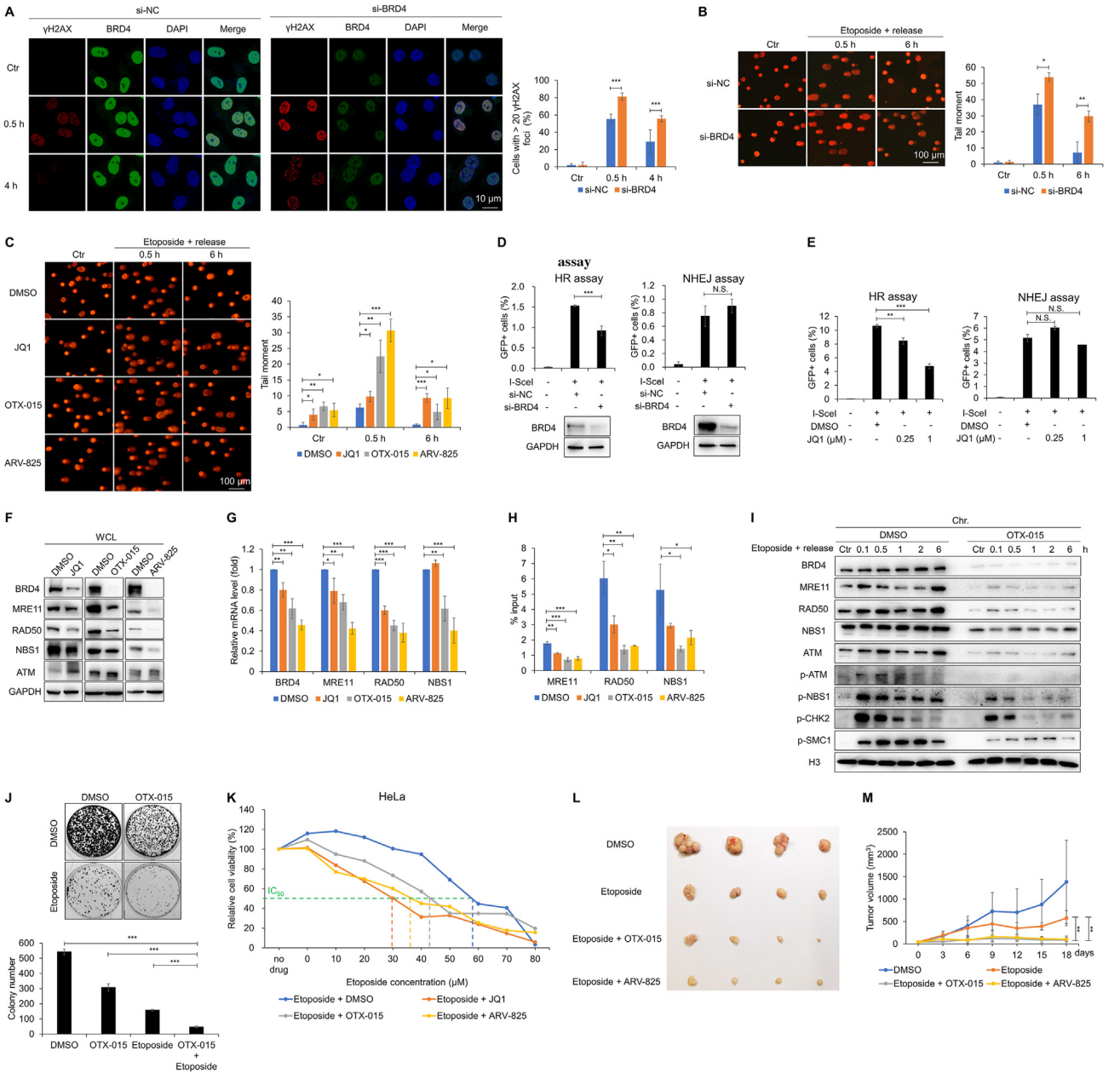


**Figure 1** BET inhibitors enhance the anti-cancer effect of etoposide by suppressing the MRN-ATM axis. **(A)** HeLa cells transfected with negative control siRNA (si-NC) or BRD4-specific siRNA (si-BRD4) for 48 h prior to treatment with 20 μM etoposide for 2 h and release for the indicated periods. The control groups were treated with an equal dosage of DMSO. Left panel: representative images of immunofluorescent analysis of cells. Right panel: percentage of cells with γH2AX foci >20 based on the numbers of γH2AX foci per cell. Scale bar = 10 μm. **(B)** Left panel: representative images of comet assays of HeLa cells transfected with si-NC or si-BRD4 for 48 h and treated with 20 μM etoposide for 1 h and following release for the indicated periods. Right panel: tail moments of each group. Scale bar = 100 μm. **(C)** Left panel: representative images of comet assays of HeLa cells treated with DMSO, 1 μM JQ1, 0.5 μM OTX-015, or 0.2 μM ARV-825 for 48 h prior to treatment with 20 μM etoposide for 1 h and following release for the indicated periods. Right panel: tail moments of each group. Scale bar = 100 μm. **(D)** HR and NHEJ assays of DR-U2OS cells (left panel) and EJ5-U2OS cells (right panel), respectively, following transfection with si-NC or si-BRD4. The bottom panel shows

wild type (WT) group, while the levels of ATM and NHEJ factors (KU70, KU80, and 53BP1) were unchanged (Fig. S2A). These findings account for the involvement of BRD4 in the HR pathway, but not the NHEJ pathway. By qRT-PCR, we showed that MRE11, RAD50, and NBS1 were down-regulated at the mRNA level (Fig. S2B). Treatment with BRD4 inhibitors also down-regulated MRE11, RAD50, and NBS1 at both the mRNA and protein levels (Fig. 1F, G), indicating that BRD4 regulates the expression of MRN complex proteins at as early as the transcription stage. Furthermore, by ChIP-qPCR assay, the enrichment of BRD4 at the promoters of *MRE11*, *RAD50*, and *NBS1* was distinctly reduced in BRD4-KO cells and inhibitor-treated cells, thus accounting for their down-regulated expression (Fig. 1H; Fig. S2C). Our findings indicated that BRD4 regulates the expression of MRN complex proteins at the transcription stage through binding to their gene promoters.

The recruitment, assembly, and activation of DDR factors at damage sites on chromatin are essential for efficient DNA repair. By Western blot, we found recruitment of MRE11, RAD50, NBS1, and ATM, as well as the phosphorylation of ATM (p-ATM) and its substrates, including p-NBS1, p-CHK2, and p-SMC1 was extensively reduced in BRD4 KO cells and inhibitor-treated cells, indicating blockade of the entire signaling pathway (Fig. 1I; Fig. S3A—C). The reduced recruitment of MRN complex proteins can be explained by their down-regulated expression caused by BRD4 deficiency and inhibition. Since the recruitment of ATM to chromatin depends partially on the MRN complex, the observed reduction in ATM recruitment and activation is most likely due to the decreased recruitment of MRN complex proteins, considering the level of ATM protein was not reduced (Fig. 1F; Fig. S2A). Therefore, BRD4 participates in the HR pathway by regulating the MRN-ATM axis.

As BRD4 inhibitors can prevent cancer cells from proper DNA repair, we speculated that they may potentially enhance the anti-cancer effect of etoposide. As expected, the colony formation assay showed that combination treatment of etoposide and BRD4 inhibitors resulted in fewer colonies than single drug treatment, indicating they could cooperatively suppress cancer cell survival (Fig. 1J; Fig. S4A, B). By MTS assay, etoposide was shown to act synergistically with all three BRD4 inhibitors to inhibit HeLa cell viability according to Valeriote and Lin's Method (Fig. S4C).[4] Moreover, we explored whether BRD4 inhibitors could be effective in etoposide-resistant cells. Among eight cell lines, NCI—H1299 and HeLa cells exhibited etoposide resistance and were less sensitive to BRD4 inhibitors, and therefore were selected for further studies (Fig. S4D—F). In both cell lines, the $IC_{50}$ values decreased markedly in combination treatment groups (Fig. 1K; Fig. S4G). Therefore, BRD4 inhibitors could sensitize etoposide-resistant cells to etoposide treatment *in vitro*.

Finally, we examined the combined effect in a nude mouse tumor xenograft model. Compared with etoposide alone, OTX-015 and ARV-825 significantly enhanced the effect of etoposide as evidenced by the smaller tumor size and lower tumor weight (Fig. 1L, M; Fig. S5A). JQ1 was not investigated due to its short half-life *in vivo*.[5] There were no significant differences in the mouse weight among the groups, suggesting that the drug combinations were tolerable in the animal model (Fig. S5B). IHC analysis revealed higher levels of cleaved caspase-3 and lower levels of Ki-67 in the drug treatment groups, indicating the occurrence of apoptosis and inhibition of cell growth (Fig. S5C). Hence, BRD4 inhibitors also sensitize cancer cells to etoposide treatment *in vivo*.

In conclusion, BET inhibitors enhance the anti-cancer effect of etoposide by suppressing the expression of MRN complex proteins, hence decreasing the recruitment and activation of the MRN-ATM axis following etoposide-induced DNA damage. As a result, DSBs caused by etoposide treatment were not efficiently repaired, leading to more cell death both *in vitro* and *in vivo* (Fig. S5D). Therefore, our study highlights BET inhibitors as important candidates for combination with etoposide to synergistically suppress cancer cells and potentially overcome etoposide resistance in chemotherapy for cancer patients, which warrants further evaluation in clinical trials.

immunoblotting of whole cell lysates (WCLs). **(E)** HR and NHEJ assays of DR-U2OS cells (left panel) and EJ5-U2OS cells (right panel), respectively, following treatment with DMSO or the indicated dosages of JQ1 for 48 h. **(F)** Immunoblotting of WCLs of HeLa cells treated with DMSO, 1 μM JQ1, 0.5 μM OTX-015, or 0.2 μM ARV-825 for 48 h. **(G)** qRT-PCR analysis of relative mRNA levels of indicated genes in HeLa cells treated with DMSO, 1 μM JQ1, 0.5 μM OTX-015, or 0.2 μM ARV-825 for 48 h. **(H)** qRT-PCR analysis of HeLa cells treated with DMSO, 1 μM JQ1, 0.5 μM OTX-015, or 0.2 μM ARV-825 for 48 h and subjected to ChIP with an anti-BRD4 antibody. **(I)** Immunoblotting of chromatin fractions extracted from HeLa cells treated with DMSO or 0.5 μM OTX-015 for 48 h prior to treatment with 20 μM etoposide for 2 h and following release for the indicated periods. **(J)** Colony formation assays of HeLa cells treated with DMSO or 0.5 μM OTX-015 for 48 h prior to treatment with DMSO or 10 μM etoposide for 1 h; representative images are shown in the top panel. **(K)** MTS assays of HeLa cells treated with DMSO, 5 μM JQ1, 1 μM OTX-015, or 0.5 μM ARV-825 in combination with etoposide at a range of dosages. $IC_{50}$ values are indicated. **(L)** HeLa xenografts collected from mice receiving different treatments at the time of sacrifice. **(M)** Tumor volume curves of HeLa xenografts from mice receiving different treatments. All data represent the mean ± standard deviation.

## Ethics declaration

The animal studies in this article were approved by the Animal Ethical and Welfare Committee of Shenzhen University.

## Author contributions

Z.L. and W.X. carried out the experiments. J.Z. and W.Z. advised on experimental design. Z.L. and W.Z. drafted the manuscript. All authors participated in the analysis and interpretation of data. All authors read and approved the final manuscript.

## Conflict of interests

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.12.001.

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