Synergistic activity of BET protein antagonist-based combinations in Mantle Cell Lymphoma cells sensitive or resistant to ibrutinib

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**Key Points:**

Bromodomain antagonist (BA) reduces MYC, CDK4/6, nuclear RelA and BTK expression and is synergistically lethal with ibrutinib in MCL cells.

Co-treatment with BA and inhibitor of BCL2, CDK4/6 or histone deacetylases is synergistically lethal against ibrutinib-resistant MCL cells.

**ABSTRACT:**

Mantle Cell Lymphoma (MCL) cells exhibit increased B cell receptor and NFκB activities. The BET protein BRD4 is essential for the transcriptional activity of NFκB. Here, we demonstrate that treatment with the BET protein bromodomain antagonist (BA) JQ1 attenuates MYC and CDK4/6, inhibits the nuclear RelA levels and the expression of NFκB target genes including Bruton’s Tyrosine Kinase (BTK) in MCL cells. While lowering the levels of the anti-apoptotic BCL2 family proteins, BA treatment induces the pro-apoptotic protein BIM and exerts dose-dependent lethality against cultured and primary MCL cells. Co-treatment with BA and the BTK inhibitor ibrutinib synergistically induces apoptosis of MCL cells. Compared to each agent alone, co-treatment with BA and ibrutinib markedly improved the median survival of mice engrafted with the MCL cells. BA treatment also induced apoptosis of the in vitro isolated, ibrutinib-resistant MCL cells which overexpress CDK6, BCL2, Bcl-xL, XIAP and AKT, but lack ibrutinib resistance-conferring BTK mutation. Co-treatment with BA and panobinostat (pan-histone deacetylase inhibitor) or palbociclib (CDK4/6 inhibitor) or ABT-199 (BCL2 antagonist) synergistically induced apoptosis of the ibrutinib-resistant MCL cells. These findings highlight and support further in vivo evaluation of the efficacy of the BA-based combinations with these agents against MCL, including ibrutinib-resistant MCL.

**INTRODUCTION**

Among the genetic alterations described in Mantle Cell Lymphoma (MCL) cells are those that involve p53, CDK4, CDKN2A, MYC, BCL2, B cell receptor (BCR) and NFκB signaling genes\(^1\)-\(^3\). These genetic alterations confer a cell autonomous pro-growth and pro-survival advantage on the MCL cells, which is especially dependent on NFκB, BCL2 and MYC activities\(^2\)-\(^4\). Next generation sequencing (NGS) has also disclosed new targets for therapeutic intervention in the deregulated molecular signaling through B cell receptor (BCR), Toll-like receptor (TLR), NOTCH, NF-κB and MAPK signaling pathways in the
MCL cell lines and patient-derived primary MCL\textsuperscript{3,4-7}. Pre-clinical and clinical studies have shown that ibrutinib, a selective, orally bioavailable, irreversible inhibitor of BTK in the BCR, also inhibits NF\kappa B activity, and is active against B-cell neoplasms, including CLL and MCL\textsuperscript{6,8}. Ibrutinib has demonstrated impressive clinical efficacy and is approved for the treatment of CLL and MCL\textsuperscript{9-11}. Despite its high level of clinical activity, primary or acquired clinical resistance to ibrutinib therapy is commonly observed\textsuperscript{11-14}. Similar to what has been described in CLL cells, a cysteine-to-serine (C481S) mutation in BTK at the binding site of ibrutinib, which results in a protein that is only reversibly inhibited by ibrutinib, has also been documented in MCL patients that relapsed while on ibrutinib\textsuperscript{12-14}. However, none of these ibrutinib-resistance associated mutations were detectable in the primary pre-ibrutinib treatment MCL tumor samples\textsuperscript{15}. Instead, mutations in MLL2, CREBBP, PIM1 and ERB4 were detected in the ibrutinib-refractory MCL cells\textsuperscript{13,15}. Additionally, as compared to the cell lines sensitive to ibrutinib exhibiting chronic activity of the classical NF\kappa B signaling pathway, ibrutinib-resistant MCL cell lines and primary MCL cells exhibited mutations in TRAF2/3 and MAP3K14 (NIK), activating the alternative NF\kappa B signaling, which would still show dependency on the NF\kappa B-activated ‘transcriptome’ for growth and survival\textsuperscript{7,16}. The deregulated transcriptome in these cells would also be governed by the genetic alterations and epigenetic mechanisms that control the expressions of MYC, BCL2 and the G1 checkpoint proteins\textsuperscript{3,7,16,17}.

Acetylation-deacetylation of the histone proteins regulates the transcriptome in transformed cells\textsuperscript{18}. The BET (bromodomain and extra-terminal) family of ‘reader’ proteins, including BRD2 (bromodomain 2), BRD3 and BRD4 recognize and bind to the acetylated lysine residues on the histone proteins associated with the open, transcriptionally permissive chromatin through their N-terminal double, tandem, 110 amino acids-long bromodomains\textsuperscript{19-21}. BET proteins also contain the extra terminal (ET) protein-interacting domain in the C-terminus which assembles a complex of co-regulatory proteins at the enhancers and promoters thereby regulating gene transcription\textsuperscript{20,21}. The C-terminal PID domain (pTEFb interacting domain) of BRD4 interacts with and recruits the pTEFb (positive transcription elongation factor b) to the ‘super-enhancers’ and promoters, thereby regulating the activity of RNA pol II (RNAP2) and gene expressions of important MCL-relevant oncogenes\textsuperscript{21-24}. Among these are MYC, CDK4/6, cyclin D1 and BCL-2, which control the proliferation and survival of MCL cells\textsuperscript{22-24}. pTEFb, which is a heterodimer composed of cyclin T and CDK9, phosphorylates Ser-2 on the heptad repeats of the CTD in the stalled RNAP2 at the transcriptional start sites, enabling the pause-release of RNAP2 and inducing productive mRNA transcript elongation\textsuperscript{24,25-27}. Thus, by promoting the availability of active pTEFb, BRD4 couples histone acetylation to transcript elongation, especially of the MCL-relevant oncogenes c-
MYC, cyclin D1, BCL-2 and CDK6\textsuperscript{21-24}. BRD4 is also essential for the transcriptional activity of NFκB triggered by the BCR signaling\textsuperscript{29,30}. BRD4 has also been shown to bind to the acetylated RelA and mediate the transcriptional activity of NFκB\textsuperscript{30}. Several structure/activity-based BET protein bromodomain antagonists (BAs) have been developed, including JQ1 and I-BET151, which displace the BET proteins, including BRD4, and the associated pTEFb, from the acetylated chromatin\textsuperscript{31-33}. This results in the transcriptional repression of BCL-2, c-MYC, cyclin D1 and CDK6, as well as, induces growth arrest and apoptosis of leukemia cells\textsuperscript{34-36}. Pertinent to this, we had previously reported that co-treatment with JQ1 and histone deacetylase inhibitor (HDI) is synergistically lethal against AML cells\textsuperscript{36,37}. In the present studies, we demonstrate the molecular basis of the activity of BAs against human MCL cells. Additionally, we demonstrate that co-treatment with BA and ibrutinib exerts superior in vitro and in vivo efficacy against MCL cells. We also demonstrate that against ibrutinib-resistant MCL cells, the combination of BA with HDI, CDK4/6 inhibitor, or BCL2 antagonist exerts synergistic lethality, which has therapeutic implication for developing effective combination therapies to achieve long term control of MCL.

**MATERIALS AND METHODS:**

**Reagents.** (S)-JQ1 (active enantiomer, hereafter referred to as JQ1) and its inactive enantiomer (R)-JQ1 were developed as previously described\textsuperscript{31}. Panobinostat (PS) was kindly provided by Novartis Pharmaceuticals, Inc. (East Hanover, NJ). Ibrutinib, palbociclib, ABT-199, ABT737, and carfilzomib were obtained from Selleck Chemicals (Houston, TX). I-BET151, an orally-available, imidazolonoquinoline-based inhibitor of the BET family of bromodomain-containing proteins was obtained from Xcessbio (San Diego, CA). All antibodies were obtained from commercial sources. Detailed antibody information is provided in the Supplemental Methods.

**Cell lines and cell culture.** Mantle cell lymphoma cell lines MO2058, JeKo-1, Z-138, and Mino were obtained and maintained as previously described\textsuperscript{38}. HS5 cells were obtained from ATCC (Manassas, VA). HK stromal cells were obtained and maintained as previously described\textsuperscript{39}.

**Assessment of cell proliferation and percentage of non-viable MCL cells.** MCL cells (MO2058, Mino, JeKo-1, and Z138) were plated in 24 well plates (0.25 x 10\textsuperscript{6}/mL) and treated with vehicle or JQ1 for 120 hours. Total cell numbers were measured in triplicate every 24 hours with a coulter counter.
Cells treated with JQ1 and/or ibrutinib were assessed for cell viability by PI staining and flow cytometry. All of these studies were performed, as previously described\textsuperscript{38,39}.

**RNA isolation and reverse transcription-polymerase chain reaction.** RNA was extracted from the cultured MCL cells using an RNaqueous–4PCR kit (Applied Biosystems, Foster City, CA). Purified total RNA was quantified, reverse transcribed and quantitative real time PCR analyses were performed on the resulting cDNA utilizing TaqMan probes from Applied Biosystems (Foster City, CA), as previously described\textsuperscript{39}. Relative mRNA expression in each treatment was normalized to the expression of GAPDH or Actin in each treatment and to the untreated control.

**Assessment of apoptosis of MCL cells.** To analyze synergism between JQ1 and PS or JQ1 and ibrutinib in inducing apoptosis, cells were treated at a constant ratio of the drugs for 48 hours. The percentages of apoptotic cells were determined by flow cytometry\textsuperscript{38}. The combination index (CI) for each drug combination was obtained by median dose effect of Chou and Talalay\textsuperscript{40} utilizing the combination index equation (assuming mutual exclusivity) within the commercially available software CalcuSyn (Biosoft, Ferguson, MO). CI values of less than 1.0 indicate a synergistic interaction of the two drugs in the combination.

**Chromatin immunoprecipitation and quantitative polymerase chain reaction.** MO2058 cells were treated with JQ1 for 16 hours. Chromatin immunoprecipitation and qPCR was performed as previously described\textsuperscript{38}.

**Mantle cell lymphoma xenograft.** Mino cells (5 x 10\textsuperscript{6} cells/mouse) were injected into the lateral tail vein of NOD/SCID or NSG mice which had received a pre-conditioning dose of radiation (2.5 gray) 24 hours prior to injection of cells. Detailed description is provided in the Supplemental Methods. The survival of mice is represented by a Kaplan Meier survival plot.

**Statistical analysis.** Significant differences between values obtained in a population of mantle cell lymphoma cells treated with different experimental conditions were determined using the Student’s t-test. For the in vivo mouse models, a two-tailed t-test or a Mantel–Cox Rank sum test was utilized for group comparisons. P values of < 0.05 were assigned significance.
RESULTS:

**Bromodomain antagonist (BA)-mediated growth inhibition and lethality of MCL cells.** We first determined the effects of the BA JQ1 on growth and survival of cultured and primary MCL cells. JQ1 treatment increased the % of G1 and inhibited the % of S phase cells in the cell cycle, as well as abrogated the suspension culture growth over 120 hours of the cultured MCL cells (Figure 1A-B and Supplemental Figure 1A). Treatment with JQ1, but not the inactive enantiomer R-JQ1, also dose-dependently induced apoptosis of the cultured MCL cells (Figure 1C and Supplemental Figure 1B). Treatment with I-BET151 also dose-dependently induced apoptosis of the cultured MCL cells (Figure 1D). Notably, treatment with JQ1 caused loss of viability of primary MCL cells purified from the lymph nodes of patients with MCL (Figure 1E). As shown in Figure 1F, co-culture with the bone marrow stromal HS5 fibroblast reduced JQ1-induced apoptosis of the cultured MCL cells. Primary MCL cells co-cultured with lymph node stromal HK cells also exhibited reduced cell death following treatment with JQ1 (Figure 1G).

**Treatment with BA attenuates pro-growth and pro-survival while increasing the levels of growth inhibitory gene expressions in MCL cells.** Next, we determined that treatment with JQ1 reduced the occupancy of BRD4 on the previously reported enhancer and promoter of MYC, and on the promoter of BCL2 in MO2058 cells (Figure 2A)\(^{36,37}\). This was also associated with a reduced occupancy of RNAP2 on the promoters of MYC and BCL2 (Figure 2B). Based on this, we evaluated the alterations in the mRNA levels in the MCL MO2058 cells, utilizing gene expression microarray (GEM) analysis (Figure 2C and Supplemental Figure 2A). Following treatment of MO2058 cells with JQ1 for 8 hours, the expression levels of c-MYC, CDK4, BCL2 and CDK6 declined while HEXIM1 levels increased (Figure 2C). In addition, the heat map of the gene expression alterations showed that the mRNA expression of more genes was increased, as compared to the number of genes whose mRNA expression was down regulated (Supplemental Figure 2A). The fold-change of the thirty most altered mRNA expressions is shown in Supplemental Table 1. Datasets of genes with the altered expression profile derived from the GEM analyses were imported into the Ingenuity Pathway Analysis (IPA) Tool (Ingenuity H Systems, Redwood City, CA)\(^{39}\). Within this gene list, IPA identified the top-five most perturbed gene networks in the MO2058 cells following treatment with JQ1 and assigned a score for these associated network functions (Supplemental Table 2). The score (e.g., a score of 57) assigned by the IPA indicates the probability (1 in \(10^{37}\)) that the focus-genes in the dataset are grouped together in a perturbed network.
due to random chance alone. Additionally, the most differentially expressed genes were assessed for pathway enrichment utilizing KEGG/Reactome pathway analysis. The top 20 significantly altered pathways in MO2058 cells identified following JQ1 treatment are listed in the Supplemental Table 3. These two distinct analyses show similar pathway perturbations due to JQ1 treatment. Next, the qPCR analysis utilizing c-MYC, CDK6 and BCL2-specific TaqMan real-time PCR probes showed that JQ1 treatment attenuated the mRNA expression of c-MYC, CDK6 and BCL2 in the MO2058 cells (Figure 2D). In contrast, JQ1 treatment induced the mRNA expression of HEXIM1 in the MCL cells (vide infra). Similar effects of JQ1 were also observed in the MCL Mino cells (data not shown). Western analysis of the protein lysates showed that treatment with JQ1 also reduced the protein expressions of c-MYC, CDK4/6, BCL2 and MCL1, while simultaneously inducing the protein expression of HEXIM1, p21, p27 and BIM in MO258 cells (Figure 2E). Similar effects of JQ1 or I-BET151 on the expression of these proteins were also documented on the cultured MCL JeKo-1 and Mino cells (Supplemental Figure 2 B-E and data not shown).

**BA treatment inhibits NFκB activity and reduces BTK levels in the cultured MCL cells.** Next, studies utilizing confocal immunofluorescence microscopy demonstrated that JQ1 treatment markedly attenuated the nuclear levels of RelA in the Mino and MO2058 cells (Figure 3A and Supplemental Figure 3A). The levels of RelA were also reduced in the purified nuclear versus cytoplasmic protein extracts of JQ1-treated versus the control Mino cells (Figure 3B). Treatment with JQ1 dose-dependently inhibited the mRNA expression of a number of the known NFκB activated, pro-growth and pro-survival genes, including TNFAIP3, cFLIP, cIAP2, XIAP, BCL-xL, BMI, IL10, PRDM1 and NFκB26,7 (Figure 3C and Supplemental Figure 3B). In contrast, JQ1 simultaneously induced the expression of HEXIM1 in the MCL cells (Figure 3C). Treatment with JQ1 also attenuated the protein levels of the anti-apoptotic XIAP and Bcl-xL proteins in the MCL cells (Figure 3D and Supplemental Figure 3C)41. BTK is also known to be activated by NFκB and shuttles between the nucleus and cytoplasm42,43. Consistent with this, JQ1-mediated NFκB inhibition attenuated BTK levels both in the nucleus and cytoplasm (Figure 3B), as well as reduced the p-BTK levels in the Mino cells (Figures 3B and 3D). Similar effects of JQ1 were observed in MO2058 cells (Figure 3D). Treatment with I-BET151 also reduced BTK and p-BTK levels in Mino cells (Supplemental Figure 3D). Notably, co-treatment with the proteasome inhibitor carfilzomib did not restore JQ1-mediated attenuation of BTK and MYC levels, thereby arguing against their degradation by the proteasome (Supplemental Figure 3E).
Co-treatment with BA and ibrutinib is synergistically lethal with concomitant depletion of BTK, nuclear RelA and NFκB activity in MCL cells. First, we confirmed that exposure to ibrutinib inhibited p-BTK levels and the downstream signaling, as well as attenuated the nuclear levels of RelA in MCL cells (Supplemental Figure 4A and 4B). Ibrutinib (10 µM for 8 hours) inhibited the mRNA levels of the NFκB-activated genes IL10, TNFa, TNFAIP3, IkBa, cIAP2, c-FLIP, PRDM1, Bcl-xL and BMI1, and induced apoptosis of MCL cells (Supplemental Figure 4C and 4D). Figure 4A demonstrates that, more so than treatment with each agent alone, co-treatment with JQ1 and ibrutinib markedly inhibited p-BTK, p-PLCγ2 and p-AKT levels in the MCL cells. Further, as compared to treatment with either agent alone, co-treatment with JQ1 and ibrutinib markedly reduced the nuclear levels of RelA in Mino cells (Figure 4B and 4C). Similar effect was also observed in MO2058 cells, along with a marked reduction in the mRNA levels of the NFκB-activated genes (Supplemental Figure 5A to 5C). Notably, combined treatment with JQ1 and ibrutinib synergistically induced apoptosis of the cultured MCL cells (with combination indices less than 1.0) (Figure 4D and Supplemental Figure 6A). JQ1 and ibrutinib was synergistically lethal against primary MCL cells obtained from patients untreated with ibrutinib, where the combination indices were lower than those observed in the cultured MCL cells (Figure 4E). This was not observed in CD19+ normal B cells (Supplemental Figure 5E). In the primary MCL cells, co-treatment with JQ1 and ibrutinib also markedly reduced p-BTK and BTK levels (Figure 4F).

Superior in vivo activity of co-treatment with BA and ibrutinib against human MCL cells. We next determined the in vivo anti-MCL activity of JQ1 and/or ibrutinib against Mino cell xenografts in the NOD/SCID mice. Seven days after tail vein infusion and engraftment of Mino cells, treatment with vehicle alone or JQ1 and/or ibrutinib was started. In Figure 5A, the Kaplan Meier plot depicting the survival of the mice demonstrates that, as compared to the treatment with vehicle alone, treatment with JQ1 or ibrutinib significantly improved the median survival of the mice (vehicle vs JQ1, p=0.003, t-test; vehicle vs ibrutinib, p=0.0204, t-test). However, co-treatment with JQ1 and ibrutinib significantly improved the median survival over treatment with the each agent or the vehicle control alone (p = 0.0013, Mantel-Cox Rank Sum), with approximately 40% of the mice treated with the combination surviving more than 50 days after the MCL cell engraftment (Figure 5A). In cohorts of three mice treated with the vehicle control versus treatment with JQ1 and/or ibrutinib for 5 days, the cell lysates of the bone marrow and spleen MCL cells demonstrated that, as compared to treatment with each agent alone, co-treatment with JQ1 and ibrutinib markedly reduced the p-BTK, BTK and p-PLCγ2 protein levels, while simultaneously increasing the levels of BIM (Figure 5B).
**BA treatment is active and exerts synergistic lethality with inhibitor of histone deacetylases, CDK4/6 and BCL2 against ibrutinib-resistant MCL cells.** Next, we generated Mino/IR cells resistant to ibrutinib by culturing Mino cells in the continuous presence of escalating doses of ibrutinib. Mino/IR cells grow in the continuous presence of 30 µM of ibrutinib. Mino/IR and Mino cells displayed a similar growth rate, but Mino/IR cells exhibited significantly higher IC50 values for ibrutinib (Mino/IR: 160.6 µM vs Mino: 13.6 µM) (Supplemental Figure 7A and 7B). Sequencing of the region around nucleotide 1634 (amino acid 481) of the BTK gene in the Mino/IR cells did not reveal any mutation in the BTK gene that had been previously discovered and reported (e.g. C481S) following in vitro or in vivo exposure to ibrutinib2,4,6,14. Notably, compared to Mino, Mino/IR cells expressed higher protein levels of BTK, TEC, AKT, CDK6, XIAP, Bcl-xL and BCL2 levels, while simultaneously displaying lower NOXA, PUMA and p21 levels (Figure 6A and 6C). In Mino/IR versus Mino cells, SOX11 expression was increased, whereas PAX5, IRF4 and BLIMP1 (PRDM1) expression levels were markedly reduced (Figure 6A)44. Compared to the expression observed in Mino cells, the Mino/IR cells lacked the expression of the plasmacytic markers CD38 or CD138. (Supplemental Figure 7C). Collectively, these findings suggest that Mino/IR cells are relatively de-differentiated transformed B-cells. Importantly, treatment with JQ1 inhibited the suspension culture growth of both Mino/IR and Mino cells (Supplemental Figure 7D). JQ1 treatment also induced a similar level of apoptosis in Mino/IR, as compared to Mino cells (Figure 6B). JQ1-induced apoptosis of Mino/IR cells was associated with a reduction in the nuclear levels of RelA, as well as inhibition of the mRNA levels of BTK, CDK6 and BCL2. In contrast, JQ1 treatment induced the mRNA expression of BCL2L11 and HEXIM1 (Supplemental Figure 7E and 7F). JQ1 treatment also markedly attenuated the mRNA levels of BTK and CDK6 (Supplemental Figure 7F); however, a more modest inhibition of Bcl-xL, BCL2, and XIAP protein levels, and a lack of any effect on the protein levels of TEC, AKT, SOX11 or PAX5, was observed (Figure 6C). Furthermore, pre-treatment with JQ1 significantly enhanced the sensitivity of Mino/IR cells to those concentrations of ibrutinib in which these cells are able to grow in suspension culture (p <0.01) (Supplemental Figure 7G). Consistent with our previous observation in AML cells36,37, combined treatment with JQ1 and panobinostat (PS) synergistically induced apoptosis of the cultured as well as of primary MCL cells, with combination indices (CI) less than 1.0 by the median dose-effect isobologram analyses (Supplemental Figure 7H and 7I). Notably, as shown in Figure 6D, co-treatment with PS and JQ1 also synergistically induced apoptosis of Mino/IR cells. Next, we determined the effect of co-treatment of JQ1 with palbociclib (a CDK4 and CDK6 inhibitor) or with the BCL2 antagonist ABT-19945,46. Co-treatment with JQ1 and palbociclib or ABT737 or ABT-199 was
synergistically lethal against not only Mino but also Mino/IR cells (Figures 6E, 6F and 6G and Supplemental Figure 6B and 7J). However, co-treatment with JQ1 and ibrutinib lacked synergy against Mino/IR cells (data not shown). We next determined whether pre-treatment with PS, palbociclib and ABT-199 lowers the threshold for ibrutinib-induced apoptosis of Mino/IR cells. We did not observe any significant effect of the pre-treatment with these agents on ibrutinib-induced apoptosis of Mino/IR cells (data not shown). Notably, co-treatment with JQ1 and ABT-199 or palbociclib was also synergistically lethal against primary MCL cells (Figure 7A and 7B). Collectively, these findings demonstrate that treatment with the BA JQ1 sensitizes MCL cells to not only HDAC inhibitor but also to CDK4/6 and BCL2 antagonists.

**DISCUSSION:**

Here, we demonstrate that, by inhibiting the activity of the BET protein BRD4 and the resulting inhibition of NFκB, treatment with a BA attenuates the expression of several NFκB-activated pro-growth and pro-survival genes, including BTK, in MCL cells. This results in the BA-mediated growth inhibition and apoptosis of MCL cells. Our findings also show that co-culture with lymph node or bone marrow stromal cells reduced BA-induced lethality of MCL cells. Whereas treatment with ibrutinib is highly effective in inducing remissions in a majority of patients, primary refractoriness or relapse with resistant disease is a common outcome in patients with MCL. We also demonstrate here that co-treatment of MCL with the BA JQ1 and ibrutinib exerts synergistic in vitro lethality and in vivo activity against MCL cells. Additionally, our findings also highlight for the first time that, while treatment with JQ1 alone is also active, co-treatment with JQ1 and panobinostat (PS), palbociclib or ABT-199 is synergistically lethal against cultured MCL cells that display in vitro resistance to ibrutinib.

As reported for AML and DLBCL cells, inhibition of BRD4 by JQ1 also attenuates the levels of c-MYC, CDK4/6 and to a lesser extent BCL2 in MCL cells, which inhibits the growth and survival of MCL cells. Acetylation of the NFκB subunit RelA at lysine 310 regulates the transcriptional activity of NFκB. BRD4 has been shown to directly bind to acetylated RelA and maintain the constitutive activity of NFκB. Additionally, DNA binding by RelA leads to increased acetylation of histone H4K5/8 to which BRD4 binds. BRD4, in turn recruits pTEFb, which phosphorylates the CTD of RNAP2 stimulate the mRNA transcript elongation of NFκB-target genes. As an acetyl-lysine mimetic antagonist of BRD4, JQ1 treatment disrupts the binding of BRD4 to the chromatin as well as to RelA, undermining the ability of BRD4 to sustain the transcriptional activity of NFκB. Consistent with this, our
findings demonstrate that JQ1 depletes the constitutively active nuclear RelA and represses the NFκB-activated genes and induces apoptosis in MCL cells. Utilizing an inducible and reversible transgenic RNAi mouse model, inhibition of BRD4 was shown recently to cause reversible epidermal hyperplasia, alopecia, decreased cellular diversity and stem cell depletion in the small intestine. However, this has not been observed following in vivo treatment of immune-depleted mice with BA.

Among NFκB-activated genes inhibited by JQ1 are the pro-growth and pro-survival genes, including TNFAIP3, cFLIP, cIAP2, XIAP, BCL-xL, BMI1, IL10, PRDM1, NFκB2, as well as BTK. Importantly, simultaneous lowering of BTK levels by JQ1 and abrogation of the activity of BTK by ibrutinib results in synergistic lethality due to co-treatment with JQ1 and ibrutinib against MCL cells. This has also been shown against the activated B-cell (ABC) DLBCL, but not against the germinal center B-cell (GCB) DLBCL or multiple myeloma cell lines. Notably, compared to each agent alone, co-treatment with JQ1 and ibrutinib also significantly improved the survival and induced a plateau in the survival curve of the immune-depleted mice engrafted with human MCL. The efficacy of co-treatment with JQ1 and ibrutinib was also associated with the in vivo attenuation of BTK, p-BTK and p-PLCγ2 levels while inducing BIM levels in the MCL cells. These findings underscore the potential for promising in vivo efficacy of the combination of BA and ibrutinib against MCL. Because it also depletes BTK expression, co-treatment with BA and ibrutinib may prevent the emergence of the mutant versions of BTK that confer resistance against ibrutinib administered alone in patients with MCL.

JQ1 treatment attenuates the levels of the anti-apoptotic proteins Bcl-xL, XIAP, MCL-1, BCL2 and p-AKT, as well as upregulates the pro-apoptotic proteins p27 and BIM. MYC is known to represses BIM via miR17-92, which is reversed following JQ1-mediated inhibition of MYC. Collectively, these effects of JQ1 lower the threshold for apoptosis and sensitize MCL cells to the apoptosis induced by a variety of agents that are also individually active as single agents against MCL cells. These agents include the pan-HDAC inhibitor panobinostat, Bcl-xL and BCL2 antagonist ABT-737, BCL2-specific antagonist ABT-199, as well as the CDK4/6 antagonist palbociclib. By attenuating the levels of CDK4/6, co-treatment with JQ1 also sensitizes MCL cells to apoptosis induced by the CDK4/6 inhibitor palbociclib. Treatment with BA also induces hexamethylene bisacetamide-inducible protein (HEXIM1), which binds to and sequesters pTEFb in an inhibitory complex, thereby inhibiting the phosphorylation of serine 2 on the CTD of RNAP2 and abrogating the mRNA transcript elongation by RNAP2. Consistent with this, HEXIM1 induction due to BA treatment is likely to contribute to the growth-inhibitory and apoptotic effects of BA-based combinations against MCL cells.
Similar to the ibrutinib-resistant Mino/IR cells described here, BTK or PLCγ2 mutations were also not discovered in the primary, ibrutinib-refractory MCL\textsuperscript{7,14,15}. Instead, mutations in TRAF2/3, BIRC3 and MAP3K14 (NIK), which activate the alternative (non-canonical) NFκB activation pathway, have been documented, resulting in the dependency of the ibrutinib-resistant MCL cells on the NFκB-activated pro-growth and pro-survival genes, or on the PI3K/AKT survival signaling\textsuperscript{7,14,16}. Compared to the parental Mino cells, ibrutinib-resistant Mino/IR cells exhibit increased expression of BTK and TEC, as well as display elevated levels of the anti-apoptotic proteins BCL2, Bcl-XL, XIAP and AKT. Consistent with this, co-treatment with JQ1 and ABT-199 or ABT-737 were synergistically lethal against Mino/IR cells. Recently, the C481S missense mutation at the ibrutinib binding site of BTK was also discovered as a secondary resistance mechanism in two patients with MCL who progressed on ibrutinib after a durable response\textsuperscript{14}. This mutation was shown to be associated with increased BTK and AKT activation, which resulted in the proliferation of the resistant MCL cells driven by increased CDK4 activity. These cells were highly sensitive to palbociclib\textsuperscript{14}. Similar to this, increased expression and dependency of Mino/IR cells on CDK6 may make them sensitive to palbociclib and also susceptible to the synergistic lethality of the co-treatment with JQ1 and palbociclib. Collectively, these pre-clinical findings show promising activity of BA-based combinations, especially with ibrutinib, against MCL cells. These findings also support further in vivo testing of the combined therapy with BA and BCL2 antagonist, HDI or CDK4/6 inhibitor against ibrutinib-resistant MCL.

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**Conflict of Interest:** All authors state that they have no conflict of interest to declare.

**Author Contributions:** B. S., B. S, W. F., S.G.T.D., D.T.S, S.K. and S. S. performed in vitro experiments with cultured and primary MCL cells, and analyzed the data. B. S. performed the in vivo studies in the NOD/SCID mice. J. Q. and J. E. B. provided a critical new reagent for the studies. L.L. provided the HK stromal cells and critically reviewed the manuscript. K.R. and C.C. processed and normalized the microarray data, provided bio-informatics support and Ingenuity Pathway Analysis of the gene expression data. J. E. B. provided intellectual input for the in vitro and in vivo studies. L.Z.
provided primary MCL samples for the studies. M. W. planned experiments and critically reviewed the manuscript. K. N. B. conceptualized, planned the experiments, supervised the studies, analyzed the data and prepared the manuscript.

References:


Figure Legends

Figure 1. Treatment with the BET antagonists JQ1 and I-BET151 induces cell cycle growth arrest and lethal effects in cultured mantle cell lymphoma cells. A. Cell cycle status of MO2058 and Mino cells following 24 hours of treatment with JQ1, as indicated. Columns, mean of three independent experiments; Bars, S.E.M. B. MO2058 and Mino cells were cultured in the presence or absence of 1.0 µM of JQ1 and cell counts were measured every 24 hours for 120 hours. Lines represent the mean cell number from three experiments ± S. D. C. MO2058, JeKo-1, Mino, and Z-138 cells were treated with the indicated concentrations of JQ1 for 48 hours. The % of annexin V-positive, apoptotic cells was determined by flow cytometry. Columns, mean of three independent experiments; Bars, S.E.M. D. MO2058 and JeKo-1 cells were treated with the indicated concentrations of I-BET151 for 48 hours. Annexin V-positive, apoptotic cells were determined by flow cytometry. Columns, mean of three independent experiments; Bars, S.E.M. IC₅₀ values were calculated using GraphPad Prism (Version 5). E. Primary MCL cells were treated with the indicated concentrations of JQ1 for 48 hours. The % non-viable cells were determined by flow cytometry. Columns, mean percent loss of viability of six primary MCL samples; Bars, S.E.M. F. Mino and MO2058 cells were co-cultured with or without HS5 stromal cells and then treated with JQ1, as indicated, for 48 hours. The % apoptosis of the MO2058 and Mino or HS5 cells was determined by staining with annexin V and To-Pro-3 iodide and flow cytometry. Columns represent the mean apoptosis of three independent experiments; Bars, S.E.M. G. Primary MCL cells were co-cultured with or without HK stromal cells and then treated with JQ1 for 72 hours. The % non-viable cells were determined by PI staining and flow cytometry. * indicates loss of viability values significantly less in cells co-cultured with HK stromal cells compared to those without co-culture.

Figure 2. Treatment with JQ1 reduces BRD4 and Pol II occupancy on the promoters of c-MYC and BCL2 and depletes the mRNA expression of c-MYC and BCL2 in human MCL cells. A-B. MO2058 cells were treated with the indicated concentrations of JQ1 for 16 hours. Following this, chromatin immunoprecipitation was conducted with a BRD4-specific antibody (A) or RNA Pol II antibody (B). The ChIP’ed DNA was subjected to quantitative, real-time PCR with primers against the enhancer and promoter of c-MYC and the promoter of BCL2. The fold change was calculated using the Ct value of the ChIP DNA compared to the Ct value of the input DNA. C. MO2058 cells were treated with 1000 nM of JQ1 for 8 hours. Total RNA was extracted and utilized for gene expression analyses. A heatmap of TNFAIP3, MYC, CDK4, BCL2, CDK6 and HEXIM1 is shown. D. MO2058 cells were
treated with the indicated concentrations of JQ1 for 16 hours. At the end of treatment, RNA was isolated and reverse transcribed. The resulting cDNA was used for real-time, quantitative PCR analysis of c-MYC, BCL-2, and CDK6. The relative mRNA expression was normalized to GAPDH and compared to the untreated cells. E. Representative immunoblots of MO2058 cells treated with the indicated concentrations of JQ1 for 24 hours. Immunoblot analyses were conducted for the expression levels of c-MYC, MCL1, CDK4, CDK6, HEXIM1, p21, p27, BIM and β-actin in the cell lysates.

**Figure 3. Treatment with BET antagonist reduces nuclear expression of RELA and BTK and depletes mRNA expression of NFkB target genes in MCL cells.**

A. Confocal immunofluorescence analysis of RelA expression and cellular localization in Mino cells following treatment with JQ1 for 24 hours. Original magnification is 63X. The bar graph shows quantification of the FITC signal intensity of the JQ1 treated Mino cells relative to the untreated cells. B. Mino cells were treated with the indicated concentrations of JQ1 for 24 hours. Following this, nuclear and cytoplasmic fractions were prepared and immunoblot analyses were conducted for BTK and RelA. The localization of Lamin B served as a fraction and loading control. The graph shows the relative expression of p-BTK and BTK in the nucleus determined by densitometry and normalized against the expression of Lamin B. Representative immunoblots are shown. C. Quantitative PCR performed on cDNA from Mino cells treated with the indicated concentrations of JQ1 for 8 hours. Relative expression of each target was normalized against GAPDH. D. Immunoblot analyses were conducted on the lysates of MO2058 and Mino cells treated with JQ1 for 24 hours, as indicated. The numbers beneath the bands represent densitometry analysis performed on the blots and normalized to the β-Actin loading control.

**Figure 4. Compared to treatment with either agent alone, combined treatment with JQ1 and ibrutinib exerts synergistic lethal activity against cultured and primary MCL cells.**

A. Representative immunoblots from Mino cells treated with JQ1 and/or ibrutinib, as indicated, for 24 hours. The numbers beneath the bands represent densitometry analysis performed on the blots and normalized to the β-Actin loading control. B. Mino cells were treated with JQ1 and/or ibrutinib for 24 hours. Confocal immunofluorescence microscopy was performed for RelA subcellular localization. Nuclei were stained with DAPI. Original magnification is 63X. C. Nuclear and cytosolic fractions were prepared from Mino cells treated as indicated for 24 hours and the expression levels of RelA in each fraction were determined by immunoblot analyses. The localization of Lamin B served as a fraction and
loading control. D. MO2058, JeKo-1 and Mino cells were treated with JQ1 and ibrutinib at a constant ratio for 48 hours. The % apoptotic cells were determined by flow cytometry. Median dose effect and isobologram analyses were performed utilizing CalcuSyn. Combination index (CI) values less than 1.0 indicate a synergistic interaction of the two agents in the combination. Doses of drugs, fractional effect and CI values are provided in Supplemental Figure 6A. E. Primary MCL cells were treated with JQ1 and ibrutinib at a constant ratio for 48 hours. The % non-viable cells were determined by flow cytometry. Median dose effect and isobologram analyses were performed utilizing CalcuSyn. CI values less than 1.0 indicate a synergistic interaction of the two agents in the combination. F. Primary MCL cells were treated with the indicated concentrations of JQ1 and/or ibrutinib for 24 hours. Then, total cell lysates were prepared and immunoblot analyses were conducted as indicated. The numbers beneath the bands represent densitometry analysis performed on the blots and normalized to the β-Actin loading control.

Figure 5. Compared to either single agent alone, co-treatment with JQ1 and ibrutinib exerts superior in vivo anti-MCL activity against Mino xenografts. A. NOD/SCID mice (n=8 per cohort) were injected with Mino cells and monitored for 7 days. Following engraftment, mice were treated with JQ1 and/or ibrutinib for 3 weeks as described in Supplemental Methods. Survival of the mice is represented by a Kaplan Meier plot. Vehicle vs JQ1 p=0.003; vehicle vs ibrutinib p=0.0204. Mantel-Cox rank sum of all groups p=0.0013. B. Immunoblot analyses conducted on the spleen and bone marrow from NOD/SCID mice injected with Mino cells as above and treated with JQ1 and/or ibrutinib for 1 week. Vertical line(s) have been inserted to indicate a repositioned gel lane.

Figure 6. Co-treatment with JQ1 and PS or palbociclib is synergistically active in ibrutinib-resistant, Mino/IR cells. A. Representative immunoblots of basal protein expression in Mino versus Mino/IR cells. B. Mino and Mino cells with acquired resistance to ibrutinib (Mino/IR) were treated with the indicated concentrations of JQ1 for 48 hours. The % of annexin V positive, apoptotic cells were determined by flow cytometry. C. Immunoblot analyses of Mino and Mino/IR cells treated for 24 hours with JQ1 and/or ibrutinib, as indicated. The numbers beneath the bands represent densitometry analysis. D-G. Mino-IR cells were treated with a fixed ratio of JQ1 and PS, or palbociclib, or ABT-737, or ABT-199 for 48 hours. The % apoptotic cells were determined by flow cytometry. Median dose effect and isobologram analyses were performed. CI values less than 1.0 indicate a synergistic interaction of the
two agents in the combination. Doses of drugs, fractional effect and CI values are provided in Supplemental Figure 6B.

**Figure 7. Cotreatment with JQ1 and ABT-199 or palbociclib is synergistically active against primary MCL cells.** A-B. Primary MCL cells were treated with the indicated concentrations of JQ1 and the BCL2-specific inhibitor, ABT-199 or the CDK4/6 inhibitor, palbociclib at a fixed ratio for 48 hours. At the end of treatment, cells were washed with 1X PBS and stained with propidium iodide. The % of non-viable cells was determined by flow cytometry. Median dose effect and isobologram analyses were performed. CI values less than 1.0 indicate a synergistic interaction of the two agents in the combination.
Figure 2
Figure 3

(A) Immunofluorescence images showing the effects of Mino and JQ1 on cell morphology and DAPI staining. "Control" and "1 µM JQ1" conditions are compared.

(B) Western blot analysis of Mino-treated cells showing the expression of cytoplasmic and nuclear proteins, including BTK, p-BTK (Y223), RelA, Lamin B1, and β-actin.

(C) Graph depicting the relative mRNA expression levels of various genes (cMyc, IL10, NF-κB2, PRDM1, TNFα, TNFAIP3, BCL-xL, BMI1, cIAP2, cFLIP, XIAP, BTK, Hexin1) under control and JQ1-treated conditions (250 nM, 1000 nM, 2000 nM).

(D) Western blot analysis showing the relative levels of p-BTK (Y223), BTK, BCL-xL, and XIAP in Mino-treated cells at 0, 250, 1000, and 2000 nM JQ1 after 24 hours.
Figure 5

A

Mino xenograft

% survival

0 20 40 60

Days

Veh vs JQ1 p = 0.003
Veh vs ibrutinib p = 0.0204
Mantel-Cox Rank Sum (all groups) p = 0.0013

B

Mino xenografts

<table>
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<tr>
<th>Spleen</th>
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<td>Control</td>
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- p-BTK (Y223)
- BTK
- p-PLCγ2
- PLCγ2
- BIM<sub>EL</sub>
- BIM<sub>L</sub>
- BIM<sub>S</sub>
- β-Actin
Figure 6
Synergistic activity of BET protein antagonist-based combinations in mantle cell lymphoma cells sensitive or resistant to ibrutinib