Inhibition of bromodomain proteins for the treatment of human diffuse large B-cell lymphoma

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Translational Relevance

JQ1 is a small molecule inhibitor of the bromodomain and extra-terminal (BET) family of bromodomain proteins. Recent studies have found anti-proliferative and pro-apoptotic effects of JQ1 in several types of malignancies. In our preclinical evaluation of JQ1 in treatment of human diffuse large B-cell lymphoma (DLBCL), we found that JQ1 has a broad antitumor activity in human DLBCL cells regardless of their molecular subtypes. JQ1 treatment resulted in either cell death or cell senescence in human DLBCL cells and led to suppression of c-MYC expression. We showed that JQ1 significantly suppressed growth of DLBCL cells engrafted in mice and improved survival of tumor-bearing mice, demonstrating a potential use of JQ1 in DLBCL treatment. We speculate that the addition of JQ1 as a component of salvage therapy or even potentially added to the standard R-CHOP therapy may result in the effective treatment of DLBCL.
Abstract

Purpose: Approximately 50% of patients with diffuse large B-cell lymphoma (DLBCL) enter long-term remission after standard chemotherapy. DLBCL patients who do not respond to chemotherapy have few treatment options. There remains a critical need to identify effective and targeted therapeutics for DLBCL.

Experimental Design: Recent studies have highlighted the incidence of increased c-MYC protein in DLBCL and the correlation between high levels of c-MYC protein and poor survival prognosis of DLBCL patients, suggesting that c-MYC is a compelling target for DLBCL therapy. The small molecule JQ1 suppresses c-MYC expression through inhibition of the bromodomain and extra-terminal (BET) family of bromodomain proteins. We investigated whether JQ1 can inhibit proliferation of DLBCL cells in culture and xenograft models in vivo.

Results: We show that JQ1 at nanomolar concentrations efficiently inhibited proliferation of human DLBCL cells in a dose-dependent manner regardless of their molecular subtypes, suggesting a broad effect of JQ1 in DLBCL. The initial G1 arrest induced by JQ1 treatment in DLBCL cells was followed by either apoptosis or senescence. The expression of c-MYC was suppressed as a result of JQ1 treatment from the natural, chromosomally-translocated or amplified loci. Furthermore, JQ1 treatment significantly suppressed growth of DLBCL cells engrafted in mice and improved survival of engrafted mice.

Conclusion: Our results demonstrate that inhibition of the BET family of bromodomain proteins by JQ1 has potential clinical utility in the treatment of DLBCL.
Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive subtype of non-Hodgkin’s lymphoma, accounting for 30-40% of all diagnoses (1). Approximately 50% of DLBCL patients enter long-term remission after R-CHOP therapy which includes a combination of chemotherapeutic agents (doxorubicin, cyclophosphamide and vincristine), prednisone, and the anti-CD20 monoclonal antibody rituximab. The inclusion of rituximab, which ablates B cells, improves the cure rate of DLBCL by 10-15% (2), indicating the benefit of targeted therapeutics. However, patients who do not respond to R-CHOP therapy or relapse soon after treatment have poor prognosis (2), and outside of possible stem cell transplantation, are left with few treatment options (3). Furthermore, DLBCL occurs mostly in elderly patients with an average age of mid-60s at diagnosis, who often have confounding factors diminishing their tolerance of chemotherapy and are often not able to tolerate intensive therapy such as transplantation. Thus, it is important to develop new targeted therapies to decrease relapse and increase the efficacy of current therapy.

DLBCL is heterogeneous with significant variations in morphology, clinical presentation and response to treatment (4). Gene expression profiling identifies biologically and clinically distinct molecular subtypes of DLBCL: activated B-cell-like (ABC), germinal center B-cell-like (GCB), and primary mediastinal B-cell lymphoma (PMBL) (5-9). These subtypes differ in molecular features and cell of origin, each arising from distinct molecular processes that ultimately lead to neoplastic transformation. Chromosomal translocations of \textit{BCL2} or \textit{c-MYC} and mutations in \textit{EZH2} are more common in the GCB subtype (6, 10-12), whereas alterations in \textit{BLIMP1/PRDM1} (13-15)
and various mutations leading to activation of the NF-κB pathway (16-20) are associated with the ABC subtype. Common to both GCB and ABC subtypes are BCL6 translocations (21, 22) and mutations in CREBBP and EP300 (23). Additional recurrent mutations in DLBCL have been identified recently (23-27), although the functional consequence of these mutations in lymphomagenesis has not been studied experimentally. Once identified, these genetic alterations provide promising targets for developing new therapeutics. In particular, a number of reports recently have highlighted the incidence of both c-MYC translocations (8.8-11%) and increased protein levels of c-MYC (29-31.8%) in DLBCL (28, 29). Moreover, high level of c-MYC protein is an adverse prognostic factor of DLBCL (28), suggesting that treatments targeting c-MYC may be beneficial clinically for DLBCL patients.

JQ1 is a small molecule inhibitor of the bromodomain and extra-terminal (BET) family of bromodomain proteins, with the highest affinity for BRD4 (30). BRD4 is a scaffolding factor that associates with acetylated chromatin to facilitate active transcription. JQ1 competitively interacts with BRD4, thus preventing BRD4 from binding to chromatin (30). Lovén and colleagues found that BRD4 preferentially occupies enhancers and super-enhancers in cancer cells, thereby increasing expression of genes already selected for expression (31). They proposed that JQ1-mediated inhibition of BRD4 preferentially decreases expression of oncogenes which have been highly selected for in any specific cancer (31). A recent study revealed highly asymmetric loading of BRD4 at super-enhancers in DLBCL cells. These super-enhancers and genes that they regulate are particularly sensitive to JQ1 inhibition, explaining the selective effect of JQ1 on oncogenic and lineage-specific transcriptional
circuits (32). One such example is the \textit{c-MYC} oncogene, which has been shown in many hematopoietic malignancies to be necessary for response to JQ1 (33-37). This led us to hypothesize that JQ1 treatment in DLBCL cells would result in decreased cell proliferation and viability in a c-MYC-dependent manner. We show here that human DLBCL cells were sensitive to JQ1 treatment in culture. These cells underwent G1 cell cycle arrest followed by either apoptosis or senescence. JQ1 treatment led to suppression of c-MYC expression, suggesting a c-MYC-dependent inhibition by JQ1. Furthermore, we found that JQ1 treatment significantly inhibited tumor growth and improved survival of mice with transplanted DLBCL cells in xenograft models.

\textbf{Materials and Methods}

\textbf{Cell lines and cell culture}

Human DLBCL cell lines OCI-Ly18, RC-K8 and SU-DHL-5 were provided by Dr. John Manis at Harvard Medical School, HBL-1, HLY-1 and OCI-Ly8 were provided by Dr. Louis Staudt at National Cancer Institute and OCI-Ly3, OCI-Ly10, SU-DHL-4 and SU-DHL-6 were provided by Dr. Subbarao Bondada at University of Kentucky. These cells lines were not authenticated independently. DLBCL cells were maintained in RPMI media (Life Technologies) plus 10\% fetal bovine serum (FBS, Sigma). Human cervical cancer cell line HeLa (ATCC) was cultured in DMEM media (Life Technologies) plus 10\% FBS. All cells were cultured at 37°C and 5\% CO₂. JQ1 as described previously (30) was dissolved in DMSO (Corning) and added in media to treat cells. JQ1 or DMSO was replaced every 48 hours with fresh media to maintain the same concentrations. After 7 days of treatment, JQ1 was removed by spinning cells at 1500 RPM for 5 minutes and
replating cells in fresh media.

**Analyses of cell viability, cell cycle, and apoptosis**

To assess cell viability, cells were collected and re-suspended in staining media: Hanks’ balanced salt solution (Life Technologies), 3% FBS, 0.2% sodium azide, 1 mM EDTA and 1 μg/ml propidium iodide (PI). Viable cell numbers were determined using a MACSQuant analyzer (Miltenyi Biotech). For cell cycle analysis, cells were collected, re-suspended in PBS, permeabilized and fixed with 95% ice cold ethanol overnight. PI was added before analyzing samples by flow cytometry using a FACSCalibur (BD biosciences). Flow cytometry data were analyzed using FlowJo software (Treestar). Apoptosis was analyzed using a Caspase-GLO 3/7 kit with GloMax-96 microplate luminometer (Promega) following manufacturer’s recommendation.

**Senescence associated β-galactosidase (SA-β-gal) staining**

Cells were collected and suspended in fixation solution (2% formaldehyde and 0.2% glutaraldehyde in PBS) for 5 min. After washing in PBS, cells were stained in SA-β-gal staining solution (40 mM citric acid/phosphate buffer, pH 6.0, 5 mM potassium ferrocynide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂, 1 mg/ml X-gal). After staining overnight at 37°C, cells were cytospun onto coverslips and examined by light microscopy.

**Western blots**

Whole cell lysates were isolated using RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM
NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid and 0.02% sodium azide) plus fresh protease inhibitor complete (Roche). Lysates were run on SDS–PAGE Criterion X-gel (Bio-Rad) and transferred to nitrocellulose membranes (GE Osmonics). Membranes were probed with antibodies against BCL-XL, Rb (Cell Signaling), c-MYC, p21, p53, β-actin (Santa Cruz Biotechnology), and p16 (Abcam). Membranes were visualized using Western lightening chemiluminescence detection (PerkinElmer) and ChemiDoc MP System with Image lab software (Bio-Rad).

**Mouse studies**

All mouse studies were carried out according to guidelines approved by the Institutional Animal Care and Use Committee of University of Massachusetts Medical School. 2 to 4-month old male NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice (Jackson Laboratory) were maintained on a bi-weekly regime of antibiotic water (400 μg/ml of sulfamethoxazole and 80 μg/ml of trimethoprim oral suspension from HiTech Pharmacal). For tumor engraftment studies, 5X10<sup>6</sup> cells suspended in 50% matrigel (BD Biosciences) were injected subcutaneously (SC) into the hind flank of each mouse (two sites per mouse). Tumors were measured using a digital caliper three times weekly for 21-days or until sacrifice. Tumor volumes were calculated using a formula: \( \frac{4}{3}\pi r_1 r_2 r_3 \), where \( r_1, r_2, r_3 \) are the radii for three dimensions of tumor. For survival studies, 5 X10<sup>6</sup> cells were injected intraperitoneally (IP). After detection of tumor by palpation (SC injected) or 6 days after tumor cell injection (IP injected), tumor-bearing mice were randomized and treated with daily IP injection of JQ1 (50 mg per kg of mouse body weight) for 21-days or until tumor volume reached 1000 mm<sup>3</sup> or mice became moribund. JQ1 were first dissolved in
DMSO and subsequently mixed with 10% hydroxypropyl-β-cyclodextrin (Sigma) to improve solubility. Vehicle treated mice were injected with the equivalent volume of DMSO mixed with 10% hydroxypropyl-β-cyclodextrin. Tissues were harvested 2 hours after injection with JQ1 or vehicle in 10% neutral-buffered formalin for paraffin sections. Tissue sections were stained with Hematoxylin & Eosin.

**Statistical analyses**

Data were presented as mean ± SD. Welch’s *t*-test and two-way ANOVA were used for statistical analyses, with *P*<0.05 considered as statistically significant. Kaplan-Meier survival curves were plotted and analyzed with the log-rank test.

**Results**

**Human DLBCL cells are sensitive to JQ1**

Recent studies found anti-proliferative and pro-apoptotic effects of JQ1, particularly in hematopoietic malignancies, and these effects of JQ1 are primarily mediated through inhibition of *c-MYC* (30, 33-40). Given recently reported incidence of *c-MYC* overexpression in DLBCL (28), we hypothesized that JQ1 treatment would inhibit human DLBCL cell proliferation and therefore might be effective in DLBCL therapy. To test this hypothesis, we used a panel of 11 human DLBCL cell lines, including four cell lines classified as the ABC subtype [HBL-1(17), HLY-1 (41), OCI-Ly3 and OCI-Ly10 (42)] and seven cell lines classified as the GCB subtype [OCI-Ly8, OCI-Ly18, SU-DHL-4, SU-DHL-5, SU-DHL-6, SU-DHL-10 (41) and RC-K8 (27)].
We treated these 11 DLBCL cell lines with increasing doses of JQ1 for 3 days, at which time we determined the number of viable cells by propidium iodide (PI) exclusion and flow cytometry. As shown in Figure 1A, total viable cells after JQ1 treatment as a percentage of DMSO-treated control cells (set to be 100%) were decreased significantly in a dose-dependent manner in all 11 DLBCL cell lines. The JQ1 dose that led to 50% inhibition of growth in DLBCL cell lines was between 25 and 160 nM, compared to 360 nM for the less sensitive human cervical cancer cell line HeLa (37). Both the ABC and GCB subtypes were sensitive to JQ1, suggesting a broad effect of JQ1 in inhibiting DLBCL cell proliferation. Furthermore, we treated four cell lines (ABC: OCI-Ly3; GCB: OCI-Ly8, SU-DHL-4 and SU-DHL-10) with two different doses of JQ1 (250 nM and 500 nM) and determined the number of viable cells at 2, 3, 4, and 7 days after treatment. As shown in Figure 1B, the numbers of viable cell at different time points after JQ1 treatment were significantly lower than those treated with the DMSO control, indicating an anti-proliferative effect of JQ1 in DLBCL cells.

**JQ1 treatment induces cell cycle arrest in DLBCL cells followed by either apoptosis or senescence**

In comparison to doxorubicin, which is known to induce apoptosis, the number of viable cells after JQ1 treatment deceased more slowly (Fig. 1B). To understand the mechanism underlying the anti-proliferative effect of JQ1 in DLBCL cells, we analyzed cell cycle distribution using flow cytometry at various time points after JQ1 treatment. **After 2 days of JQ1 treatment, a significant decrease in percentage of cells in the S phase and a significant increase in percentage of cells in the G1 phase of the cell cycle**
were observed (Fig. 2). Further, there was insignificant or minimal increase in the sub-G1 population (Fig. 2), suggesting that JQ1 induces cell cycle arrest with minimal cell death after 2 days of JQ1 treatment. After 4 days of JQ1 treatment, OCI-Ly8 and SU-DHL-4 cells maintained cell cycle arrest, whereas a consistent increase in the sub-G1 population was observed in OCI-Ly3 (5.5% for 250 nM JQ1-treated and 7.8% for 500 nM JQ1-treated compared to 0.7% for DMSO-treated control) and SU-DHL-10 (3.1% for 250 nM JQ1-treated and 3.5% for 500 nM JQ1-treated compared to 1.1% for DMSO-treated control) (Supplementary Fig. S1). In comparison, doxorubicin treatment led to a significant increase in the sub-G1 population in SU-DHL-10, OCI-Ly3 and OCI-Ly8 cells even after 2 days of treatment (Fig. 2 and Supplementary Fig. S1). Neither doxorubicin nor JQ1 induced a significant increase in the sub-G1 population in SU-DHL-4 cells up to 4 days of treatment (Fig. 2 and Supplementary Fig. S1). To further investigate cell death after JQ1 treatment, we measured caspase 3/7 activity as an indicator of apoptosis. We found a small but consistent increase in caspase 3/7 activity 4 days after treatment with 250 nM JQ1 compared with DMSO-treated control cells in OCI-Ly3 (2.8-fold increase) and SU-DHL-10 (1.6-fold increase) cells, whereas a significant increase in caspase 3/7 activity was observed in both OCI-Ly3 (12.9-fold increase) and SU-DHL-10 (353-fold increase) cells after 4 days of doxorubicin treatment compared to DMSO-treated controls (Fig. 3A).

Interestingly, we found that the initial G1 cell cycle arrest was followed by either apoptosis or senescence after longer (7-day) treatment with JQ1. OCI-Ly3, SU-DHL-4 and SU-DHL-10 cells showed significant increase in the sub-G1 populations: 27.5% (250 nM JQ1) and 12.2% (500 nM JQ1) compared to 1.0% (DMSO) for OCI-Ly3; 19.3%
(250 nM JQ1) and 31.6% (500 nM JQ1) compared to 2.8% (DMSO) for SU-DHL-4; 9.2% (250 nM JQ1) and 8.2% (500 nM JQ1) compared to 2.3% (DMSO) for SU-DHL-10 cells (Fig. 2). In contrast, OCI-Ly8 did not have an increased sub-G1 population, but rather maintained a G1 arrest (Fig. 2). Apoptosis in SU-DHL-4, SU-DHL-10 and OCI-Ly3 cells and the lack of cell death in OCI-Ly8 cells were corroborated by the analysis of caspase 3/7 activity. As shown in Figure 3B, a significant increase in caspase 3/7 activity was observed in SU-DHL-10, SU-DHL-4, and OCI-Ly3 cells treated with 250 nM JQ1 compared to DMSO control (16-fold, 20-fold and 6.2-fold increase, respectively). In comparison, OCI-Ly8 showed only a small increase (2.4-fold increase) in caspase 3/7 activity after 7 days of treatment with 250 nM JQ1.

To further characterize the anti-proliferative effects of JQ1, we removed JQ1 after 7 days of treatment and asked whether removal of the drug from the cells for an extended period allowed the cells to re-enter the cell cycle. All of the four cell lines tested either maintained growth arrest, or in the case of SU-DHL-4, had decreased cell numbers (Fig. 4A). This suggests that JQ1 treatment caused cells to permanently exit the cell cycle. To test whether these cells entered senescence, we stained them for senescence associated β-galactosidase (SA-β-gal) activity. After 7 days of treatment with 250 nM JQ1, we observed a significant percentage of cells staining positively for SA-β-gal in OCI-Ly3 (71.6%) and OCI-Ly8 (90.6%) cells. In contrast, only 5.4% or no SA-β-gal positive cells were observed in SU-DHL-10 and SU-DHL-4 after JQ1 treatment for 7 days (Fig. 4B). These data indicate that JQ1 treatment results in two independent phenotypes in human DLBCL cell lines: apoptosis and senescence.
JQ1 treatment leads to suppression of c-MYC expression

As JQ1-mediated suppression of c-MYC expression is responsible for its anti-proliferative effect in various types of cancer cells (33, 36, 37, 43-45), we examined c-MYC protein levels in the 11 DLBCL cell lines that were characterized for response to JQ1 treatment. In particular, OCI-Ly3 (46) and SU-DHL-4 (47) cells have c-MYC amplifications. HBL-1 (48), OCI-Ly8, OCI-Ly18 (46), SU-DHL-6, and SU-DHL-10 (47) cells have c-MYC translocations, whereas c-MYC loci in RC-K8 (49), SU-DHL-5 (47), OCI-Ly10 and HLY-1 (John Manis, personal communication) cells are normal. We found that these cell lines expressed varying amounts of c-MYC protein (Fig. 5A). After JQ1 treatment for 2 days, c-MYC protein level was clearly decreased in 10 out of 11 DLBCL cell lines that we tested (Fig. 5A). The c-MYC level did not change significantly in SU-DHL-10 cells after 2 days of JQ1 treatment (Fig. 5A). However, an 88% decrease in the c-MYC protein level was observed in SU-DHL-10 cells after 7 days of JQ1 treatment (Fig. 5B). JQ1 treatment decreased the c-MYC level in cells with c-MYC translocations (HBL-1, OCI-Ly8, OCI-Ly18, SU-DHL-6, and SU-DHL-10), c-MYC amplifications (OCI-Ly3 and SU-DHL-4) or without change in the c-MYC loci (RC-K8, SU-DHL-5, OCI-Ly10 and HLY-1). These data suggest that JQ1-mediated suppression of c-MYC can occur at the natural, chromosomally-translocated or gene amplified c-MYC loci.

To understand the possible mechanisms of the apoptosis versus senescence response after JQ1 treatment, we examined expression of proteins previously implicated in these processes. Levels of anti-apoptotic factor BCL-XL either did not change or decreased upon JQ1 treatment (Fig. 5B). In addition, proteins known to regulate senescence, p16, p21 and Rb, were either undetectable or showed decreased
expression after JQ1 treatment. Protein levels of p53 were not changed and its mutation status did not appear to correlate with response to JQ1 treatment, as OCI-Ly3 cells have wild type p53 (50) and OCI-Ly8 cells harbor a mutated p53 (46). Although p53 in SU-DHL-10 and SU-DHL-4 cells has not been annotated, the reduced p53 protein size in SU-DHL-10 suggests a truncation (Fig. 5B).

**JQ1 treatment of xenograft tumors results in significantly decreased rate of tumor growth and increased survival of mice**

To evaluate how DLBCL cells xenografted into NSG mice would respond to a regimen of treatment with JQ1, we began by engrafting OCI-Ly8 cells subcutaneously into NSG mice. When at least one tumor on each mouse was detectable by palpation, we began a daily treatment of JQ1 (50 mg/kg of mouse body weight) or vehicle for 21 days. Seven mice from the vehicle-treated group and 4 mice from JQ1-treated group had to be euthanized before the end of treatment regimen because tumor volumes reached 1000 mm³. Tumor growth was significantly decreased in JQ1 treated mice compared with vehicle treated ($P<0.001$) (Fig. 6A). In addition to subcutaneously injected tumors, we used OCI-Ly8 cells to engraft NSG mice intraperitoneally in order to better mimic human disease. Six days after cell injection, we began daily treatment with 50 mg/kg JQ1 or vehicle for 21 days. After the completion of treatment, we monitored the mice until all were moribund and therefore had to be euthanized. JQ1 significantly ($P=0.0039$) increased survival time with a mean survival of 33.6 days compared with 29.5 days for vehicle (Fig. 6B). Upon sacrifice, mice displayed infiltration of tumor cells into spleen and liver (Fig. 6C) as well as prominent abdominal masses.
Discussion

In this study, we demonstrated that targeting BRD proteins by JQ1 in human DLBCL cells resulted in cell death or cell senescence. We found that DLBCL cells were sensitive to JQ1 treatment regardless of molecular subtypes (ABC vs. GCB), suggesting that JQ1 has a broad effect in DLBCL. This broad effect is especially encouraging for the potential clinical utility of JQ1 for DLBCL treatment. Recent studies have found anti-proliferative and pro-apoptotic effects of JQ1, particularly in hematopoietic malignancies, and these effects of JQ1 are primarily mediated through inhibition of \( c-MYC \) (30, 33-40). Consistent with these findings, we showed that JQ1 mediated suppression of \( c-MYC \) expression, regardless of whether it is from un-perturbed \( c-MYC \) loci (HLY-1, OCI-Ly10, RC-K8 and SU-DHL-5), or from chromosomally-translocated (HBL-1, OCI-Ly8, OCI-Ly18, SU-DHL-6, and SU-DHL-10) or amplified (OCI-Ly3 and SU-DHL-4) loci. Collectively, our studies suggest that inhibition of \( c-MYC \) via BET bromodomain family proteins by JQ1 provides a promising therapeutic model for patients with DLBCL.

Previously it has been shown that cancer cells respond to JQ1 primarily with rapid cell cycle arrest and apoptosis (30, 33-35, 37, 38). These studies demonstrate increased apoptotic markers within 2-3 days of JQ1 treatment. We found two distinct phenotypes in response to JQ1: senescence or apoptosis. OCI-Ly8 cells displayed G1 arrest and positive staining for SA-\( \beta \)-gal without increases in sub-G1 population and apoptosis following JQ1 treatment, indicating that senescence is the main response to JQ1 in OCI-Ly8 cells. SU-DHL-4 and SU-DHL-10 cells showed little or no positive SA-\( \beta \)-gal staining but increased sub-G1 population and apoptosis, indicating that apoptosis is
the prominent response to JQ1 in these two cell lines. Both apoptosis and senescence were observed in OCI-Ly3 cells. The apoptosis vs. senescence phenotype did not correlate with the parameters that we examined, including molecular subtype (ABC: OCI-Ly3 vs. GCB: OCI-Ly8, SU-DHL-4 and SU-DHL-10), p53 mutation status (wild type: OCI-Ly3 vs. mutated: OCI-Ly8 and SU-DHL-10), c-MYC translocation and expression level (amplified: OCI-Ly3 and SU-DHL-4; translocated: SU-DHL-10 and OCI-Ly8), BCL2 translocation status [translocated: SU-DHL-4, SU-DHL-10 (47) and OCI-Ly8 (46); amplified: OCI-Ly3 (46)], or expression of p16, p21, p53, Rb or BCL-XL. A recent study revealed highly asymmetric loading of BRD4 at super-enhancers in DLBCL cells. These super-enhancers and genes that they regulate are particularly sensitive to JQ1 inhibition, explaining the selective effect of JQ1 on oncogenic and lineage-specific transcriptional circuits (32). It will be interesting to understand how this selectivity of BRD4 loading at super-enhancers and inhibition by JQ1 are responsible for the different responses (apoptosis vs. senescence) to JQ1 in DLBCL cells.

We showed that JQ1 significantly suppressed growth of DLBCL cells engrafted in NSG mice and improved survival of tumor-bearing mice, demonstrating a potential use of JQ1 in DLBCL treatment. We found that JQ1 alone with the regimen tested was not sufficient to cure the disease. The differences in JQ1 effectiveness observed between in vitro and in vivo studies could be due to a number of issues, one of which is bioavailability in vivo. The half-life of JQ1 in plasma is relatively low: 0.9 hours (intravenous injection of 5 mg/kg) or 1.4 hours (oral administration of 10 mg/kg) (30). When dosed every 24 hours, it is likely that very little JQ1 remains after 8 hours. Although it has not been previously measured, the effective concentration of JQ1 at the
site of tumors is likely even lower than in the plasma, because the typically poor vasculature in tumor tissues could prevent JQ1 from being effectively delivered to the tumor cells. A recent study using 2 daily doses of JQ1 treatment for 30 days (vs. 1 daily dose for 21 days in our study) shows a median survival advantage of 9 days in NSG mice with DLBCL xenografts (32) compared to 4 days in our study, suggesting that more frequent dosing to maintain plasma concentration of JQ1 over time may increase its effectiveness in vivo, especially given that no adverse effects have been reported for JQ1 use in mouse models. Additional modifications to the structure of JQ1 that maintain its specificity while increasing the half-life will make JQ1 more effective in vivo and in clinical use.

It is possible to combine the use of JQ1 with the current standard therapy to increase the efficacy of treatment. A recent study by Emadali and colleagues showed that the addition of JQ1 to rituximab increases sensitivity of rituximab-resistant DLBCL cell lines (39). This study provides proof-of-principal that adding JQ1 to current treatments may be beneficial. Another study has demonstrated additional value of JQ1 in combination with other novel therapies for DLBCL. Zhao and colleagues show that treating DLBCL cell lines with EZH2-inhibitor DZNep and JQ1 reduces cell viability in a synergistic manner (40). We propose that JQ1 should be examined clinically in patients with DLBCL. We speculate that the addition of JQ1 as a component of salvage therapy or even potentially added to R-CHOP therapy may result in the effective treatment of DLBCL. Continued examination of JQ1 alone and in combination with other novel therapeutic agents is warranted.
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References


that controls Rituximab response and tumour growth in aggressive lymphoid cancers.


Figure Legends

Figure 1. JQ1 treatment of human DLBCL cell lines leads to decreased cell viability. (A) 12 cell lines, including 11 human DLBCL cell lines and a human cervical cancer cell line HeLa, were treated with the indicated doses of JQ1 for 3 days. Viable cells were determined by PI exclusion in flow cytometry. The number of viable cells after JQ1 treatment is normalized as percent of viable cell number of DMSO-treated control. Error bars are SD of at least 3 independent experiments. (B) Proliferation of 4 DLBCL cell lines. Cells were treated with the indicated doses of JQ1 and doxorubicin (Dox) and viable cells were determined by PI exclusion in flow cytometry at indicated time after treatment. Error bars are SD of 3 independent experiments. Two-way ANOVA was used to compare JQ1 or Dox-treated with DMSO-treated controls. ***: P<0.001.

Figure 2. JQ1 induces cell cycle arrest and cell death in human DLBCL cells. Cell cycle analyses of DLBCL cells treated with JQ1 or doxorubicin (Dox) for 2 days and 7 days were shown. Error bars are SD of 3 independent experiments. Welch’s t-test was used for statistical analysis. *: P<0.05, **: P<0.01, ***: P<0.001.

Figure 3. JQ1 induces apoptosis in human DLBCL cells. Caspase 3/7 activity in DLBCL cells treated with JQ1 or Dox for (A) 4 days or (B) 7 days was measured and normalized with that in cells treated with DMSO, which was set to be 1. Error bars are SD of 5 independent experiments. Welch’s t-test was used for statistical analysis. **: P<0.01, ***: P<0.001.
Figure 4. JQ1 induces senescence in OCI-Ly8 and OCI-Ly3 cells. (A) DLBCL cells were treated with JQ1 for 7 days. JQ1 or DMSO was then washed away and viable cells were counted by PI exclusion in flow cytometry at 3 and 5 days after drug removal. Error bars are SD of 3 independent experiments. (B) Representative staining of SA-β-gal in cells treated with JQ1 for 7 days. Percent of SA-β-gal positive cells was quantified from 5-8 randomly selected fields with at least 500 cells. Error Bars are SD and Welch’s t-test was used for statistical analysis. **: P<0.01, ***: P<0.001.

Figure 5. JQ1 treatment leads to suppression of c-MYC expression in DLBCL cells. (A) Western analysis of c-MYC protein levels in human DLBCL cell lines treated with JQ1 (250 nM) or DMSO for 2 days. The status of c-MYC in each cell line is indicated as: A (amplified), T (translocated) and N (normal). SU-DHL is abbreviated as SU. (B) Western analysis of c-MYC, BCL-XL, p16, p21, p53 and Rb protein levels in DLBCL cells treated with JQ1 (250 nM) or DMSO for 7 days.

Figure 6. JQ1 treatment suppresses tumor growth and improves survival of NSG mice engrafted with DLBCL cells. (A) Growth of OCI-Ly8 tumor cells injected subcutaneously into NSG mice. Mice were treated with JQ1 or vehicle for 21 days or until tumor volume reached ~1000 mm³. Tumor growth in each individual mouse and average of tumor growth in JQ1 or vehicle treated mice are shown. Two-way ANOVA is used for statistical analysis. ***: P<0.001. (B) Kaplan-Meier survival curve of NSG mice engrafted with OCI-Ly8 tumor cells intraperitoneally and treated with JQ1 or vehicle for 21 days. Log-rank test is used for statistical analysis. (C) H&E staining showing...
evidence of tumor infiltration into liver and spleen of two representative NSG mice (90E treated with vehicle and 91C treated with JQ1) engrafted with OCI-Ly8 cells intraperitoneally. Spleen section of a normal non-engrafted NSG mouse is shown for comparison.
**Figure 1**

(A) Viable cells compared to DMSO across different cell lines and concentrations of JQ1. The graph shows a significant decrease in viable cells with increasing JQ1 concentration.

(B) Viable cell number (x10^5) over days for different cell lines treated with DMSO, JQ1 [250 nM], JQ1 [500 nM], and Dox [172 nM]. The graph indicates a significant effect on cell viability compared to the control group.

<table>
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<th>Cell Line</th>
<th>DMSO</th>
<th>JQ1 [250 nM]</th>
<th>JQ1 [500 nM]</th>
<th>Dox [172 nM]</th>
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<td>6.25</td>
<td>-</td>
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</table>

*** Significant difference compared to control.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

A. Mean tumor volume (mm$^3$) over days of treatment. The graph shows the progression of tumor volume in different treatment groups. The data points are represented by various symbols and colors, indicating different groups.

B. Percent survival over days after tumor cell injection. The survival rate is compared between the vehicle and JQ1 treatment groups. The graph includes a statistical significance level indicated by ***.

C. Histological images of the spleen and liver from different groups. The images are captured at 10X and 50X magnification, with measurements of 500 µm and 100 µm respectively.
Inhibition of bromodomain proteins for the treatment of human diffuse large B-cell lymphoma

Sally E Trabucco, Rachel M. Gerstein, Andrew M. Evens, et al.

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