Original Article
BRD4 inhibition sensitizes aggressive non-Hodgkin lymphomas to PI3Kδ inhibitors by suppressing PI3K reactivation and c-MYC expression

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Abstract: Targeting phosphatidylinositol 3-kinase δ (PI3Kδ) is an important therapeutic strategy for indolent non-Hodgkin lymphomas (NHLs). However, we previously observed reactivation of phosphatidylinositol 3-kinase (PI3K) pathways in aggressive NHL cell lines following continuous exposure to PI3Kδ inhibitors (PI3Kδi), which limited their efficacy and suggests that more studies should be focused on this reactivation to improve current PI3Kδi-based treatments. Herein we conducted a drug synergy screening that combined a marketed PI3Kδi, idelalisib, with 14 well-characterized epigenetic drugs across several types of aggressive NHL cell lines. We identified BRD4 inhibitors (BRD4i) as potent partners that, in combination with idelalisib, were capable of synergistically exerting anti-proliferative activity and inducing cell apoptosis in a panel of aggressive NHL cell lines through continuous suppression of PI3K pathways. More importantly, the combination of BRD4i and PI3Kδi simultaneously inhibited transcription and translation of the oncogenic transcription factor c-MYC, downregulating the expression of c-MYC and continuously suppressing the proliferation of cancer cells in vitro, as well as the growth of tumors in vivo even after drug withdrawal. This study, thus, reveals the potential of simultaneously targeting PI3Kδ and BRD4 as a new therapeutic strategy for aggressive forms of NHL.

Keywords: Aggressive NHL, PI3Kδ inhibitors, drug synergy screening, BRD4 inhibitors, drug withdrawal

Introduction
Non-Hodgkin’s lymphoma (NHL) is the most common hematological malignant B-cell tumor in humans and includes both indolent and aggressive types. Despite improvements in survival owing to the approval of several monoclonal antibodies and kinase inhibitors, the survival rate for NHL patients remains low due to disease progression or recurrence [1]. Hence, there remains a need to develop safer and more effective therapeutic strategies. Activating B-cell receptor (BCR) signaling is important in the growth of lymphoid tumors derived from mature B-cells [2-4]. Meanwhile, the phosphatidylinositol 3-kinases (PI3Ks), comprising four catalytic isoforms (p110α, β, γ, and δ) and two regulatory subunits (p85 and p101), are pivotal for BCR signaling and are key driving factors in several cancers [5-8].

The δ isoform of the p110 catalytic subunit (p110δ) is highly expressed primarily in the B-cell hematopoietic system and plays an essential role in B-cell survival, migration, and activation [9, 10]. Indeed, idelalisib (GS1101/CAL-101), a PI3Kδ inhibitor (PI3Kδi), was the first agent approved by the US Food and Drug Administration (FDA) to treat, in combination with rituximab, indolent NHL (iNHL) and relapsed or refractory chronic lymphocytic leukemia (CLL) [11-13]. In contrast to its significant clinical benefit in iNHL and CLL, idelalisib was not effective against aggressive NHLs [14, 15]. Reactivation of PI3K-dependent pathways after PI3Kδi treatment was reported in several
types of malignancies, which could partially explain disease relapse [16]. Moreover, activation of the MYC proto-oncogene, bHLH transcription factor (MYC) also overrides PI3K/AKT/mTOR-dependent proliferation in multiple cancer cell lines [17]. Therefore, to improve the efficacy of PI3Kδi against aggressive NHL, two important problems must be solved: (i) preventing of reactivation of the PI3K pathway after PI3Kδi treatment by ensuring durable inhibition and (ii) downregulation of MYC expression to restore sensitivity to PI3Kδi.

PI3Kδi-based combinations, such as idelalisib and ibrutinib or rituximab, have been approved by the FDA and demonstrated significant clinical efficacy, however, only a few patients with aggressive NHL have benefited from the current combinatorial therapies. In addition, most combination strategies are in the preclinical stage or in early clinical trials [18]. Additional combinations need to be developed to benefit more NHL patients and to provide more therapeutic options.

Epigenetic proteins are fundamental components that control gene expression and have been unequivocally linked to tumorigenesis and tumor progression [19-22]. Several small molecules targeting epigenetic proteins have been reported for cancer therapy. These epigenetic modulators, which function at the gene level by regulating the expression of oncogenes and anti-oncogenes, provide us with possible strategies to address the limitations of traditional kinase inhibitors that act only on single signaling pathways. Furthermore, several clinical and preclinical studies have provided convincing evidence that the combination of epigenetic modulators and kinase inhibitors is an attractive therapeutic strategy [18, 23]. Therefore, combining epigenetic and kinase inhibitors could provide improved alternative treatment options for some patients.

The bromodomain and extra-terminal domain (BET) family of proteins (BRD2, BRD3, BRD4, and BRDT) constitute epigenetic readers that regulates gene transcription through recognizing acetylated-lysine residues in nucleosome histones, facilitating the recruitment of transcriptional factors to chromatin and subsequently regulating the transcription of cell proliferation-related genes such as MYC and MYCN [19]. BRD4i, specifically, comprises a class of epigenetic inhibitors for lymphoma, acute leukemia, and various solid tumors treatments and are used in drug combination therapies to overcome adaptive resistance of different types of cancer [24-26].

Here, we established a platform to systematically screen epigenetic inhibitors along with PI3Kδi to improve the response of aggressive NHLs to PI3Kδ inhibition. BRD4i showed a potent synergistic effect with idelalisib against aggressive NHLs, including GCB-DLBCL, MCL, and BL, that was always involved in drug combination therapies and might overcome adaptive resistance mechanisms [24-26]. The resulting combination of PI3Kδi and BRD4i was further investigated for in vitro and in vivo anticaner effects and their mechanisms of action were elucidated in different aggressive NHL models. To further validate the therapeutic advantages of this co-treatment, the in vitro and in vivo anticaner effects after drug withdrawal were also evaluated. This study provides a foundation for further investigation of PI3Kδi and BRD4i combinatorial therapy for the clinical treatment of aggressive NHL.

Materials and methods

Cell culture and preparation of compounds

All cell lines used for in vitro and in vivo experiments were purchased from American Type Culture Collection (Manassas, VA, USA) in 2017. SU-DHL-6, SU-DHL-4, JEKO-1 cells were authenticated using DNA fingerprinting short-tandem repeat (STR) assays, other cell lines were not authenticated. Cells were cultured in Rosewell Park Memorial Institute (RPMI) 1640 media supplemented with 20% fetal bovine serum (Gibco, Grand Island, NY, USA) and Penicillin-Streptomycin (Life Technologies, Carlsbad, CA, USA) under humidified conditions at 37°C, 5% carbon dioxide. Cells were cultured for less than 3 months.

All compounds were obtained from Selleckchem (Houston, TX, USA) in 2017. Inhibitors for in vitro studies were dissolved in Dimethyl Sulfoxide (DMSO) to 10 mM stock solutions and stored at -80°C.

Cell proliferation assay and drug synergism analysis

CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA)
was used according to manufacturer’s protocol. Briefly, cells were seeded (6 × 10^3-8 × 10^3 cells per well) in 96-wells plates and then treated with different doses of drugs in a total volume of 200 μL. After indicated times, 10 μL of CellTiter 96® AQueous One Solution reagent was added to the cells and incubated for 2-4 h at 37°C and luminescence was measured using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 495 nm. The IC_{50} values were calculated using the GraphPad Prism 7 software (GraphPad, Inc., La Jolla, CA, USA).

The 6*6 orthogonal design was used for analyzing the effects of drug combinations and drug synergism using CompuSyn software (version 1.0), which is based on the combination index-isobologram theorem (Chou-Talalay) and generates combination index (CI) values. Where CI≥1.4 indicates strong antagonism, CI≥1.2, <1.4 indicates antagonism, CI≥0.8, <1.2 indicates nearly additive, CI≥0.6, <0.8 indicates synergism and CI<0.6 indicates strong synergism.

**Cell apoptosis analysis**

The apoptosis assay was conducted using flow cytometry (FCM). Cells were treated with idelalisib and JQ1 alone or in combination for 24, 48, 72 h, respectively. Next, the cells were harvested and washed with PBS, and stained with an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Roche, Indianopolis, IN, USA) according to the manufacturer’s protocol. A minimum of 1 × 10^4 cells were analyzed using the BD FACSCanto™ II (BD Biosciences, San Jose, CA USA) and the data was processed with FlowJo software (V10.4, Ashland, OR, USA).

**Western blotting analysis**

Cells were treated with idelalisib and JQ1 alone or in combination for indicated time, and then harvested and washed with PBS. The harvested cells were lysed in RIPA buffer (Beyotime, Beijing, China) supplemented with cocktail (1:1000) and phosphatase inhibitors (Roche, Basel, CH) for 30 min and equalized before loading. The samples were separated by SDS-PAGE and transferred onto nitrocellulose (NC) filter membranes (Merck Millipore, MS, USA). The membranes were incubated with appropriate primary antibody overnight at 4°C, washed thrice and then incubated with corresponding secondary antibody. Chemiluminescence detection were used to detect specific protein bands. Details of the antibodies used in this article can be found in the Table S1.

**Quantitative real time-PCR (qRT-PCR) analysis**

Cells were treated with idelalisib and JQ1 alone or in combination, for 24 h. Total cellular RNA was extracted using an mRNeasy kit (QIAGEN, Valencia, CA), with DNAse treatment and RNA was reverse transcribed into cDNA with the Gene touch System (Bioer, CN), following manufacturer’s protocol (Mei 5 Biotechnology, Co., Ltd). Targeted gene expression was performed using SYBR Green RT-PCR Kit from Takara on a BIO-RAD CFX96 Real-Time PCR system, following manufacturer’s instructions. The PCR primer sequences used in this article can be found in the Table S2.

**Xenograft mouse model**

NOD-SCID (6- to 9-week-old) mice used in this study were purchased from Beijing HFK bioscience Co. Ltd (Beijing, China). Mice maintenance were performed in a specific-pathogen-free (SPF) condition facility and all animal experiments were approved by the Institutional Animal Care and Treatment Committee of Sichuan University in China (Permit Number: 20170135), which were carried out in accordance with the approved guidelines. Mice were subcutaneously inoculated with ~1 × 10^7 SU-DHL-6 or JEKO-1 cells. Once the mice developed tumors of approximately 100 mm^3, they were randomly divided into four groups: (i) Vehicle (Vehicle, oral gavage, n = 5); (ii) Idelalisib (25 mg/kg, oral gavage, n = 5); (iii) JQ1 (50 mg/kg, oral gavage, n = 5); (iv) Combination group 1 (25 mg/kg idelalisib plus 50 mg/kg JQ1, oral gavage, n = 5). The treatment groups, or the vehicle group were administered at the indicated doses once per day for 18 or 21 days. Tumor volumes were measured by electronic slide caliper every three days and calculated using the following formula: tumor volume (mm^3) = 0.52 × length × width^2. After administration, three mice were euthanized and tumors were extracted, others were monitored for 12 or 9 days, at which time mice were euthanized and tumors were extract.
ed. The tumor growth inhibition (TGI) values were calculated with the following formula: 
\[ TGI = \left[ 1 - \frac{aT_n - T_0}{C_n - C_0} \right] \times 100\% \]
where \( T_0 \) represents average tumor volume before treatment and \( T_n \) represents average tumor volume of \( n \) days after treatment. \( C_0 \) represents average tumor volume before treatment and \( C_n \) represents average tumor volume on day \( n \) after treatment in vehicle group.

Immunohistochemistry (IHC) analysis

Tumor tissues were fixed overnight in 4% paraformaldehyde and then embedded in paraffin. Sections were subjected to immunohistochemical staining with antibody following standard protocols after cut into 5 μm sections. The antibodies are listed in the Table S1. The investigator who measured Immunohistochemistry (IHC) analysis was unaware of group allocation.

Statistical analysis

The Student’s t-test was used to examine statistically significant differences between control and treatment groups. All statistical analyses were performed using SPSS software (Armonk, NJ, USA) and quantitative results are expressed as mean values ± SD. Statistically significant P-values were expressed as follows: *P<0.05; **P<0.01; ***P<0.001.

Results

Drug combination screening identified synergistic effects of BRD4i and PI3Kδi

To determine whether epigenetic regulatory molecules sensitize aggressive NHL cells to PI3Kδi, we screened drug combinations using the SU-DHL-6 DLBCL cell line, which highly expresses and modestly responds to PI3Kδ. An FDA-approved PI3Kδi, idelalisib (GS1101/CAL-101), and 14 well-characterized epigenetic inhibitors targeting seven epigenetic regulators (two compounds for each class, Figures 1A and S1) were initially screened using SU-DHL-6 cells that were treated for five days with each combination. The average combination index (CI) values of idelalisib and each epigenetic drug are summarized in Figure 1A. Two BRD4i (JQ1 and OTX015) showed strong synergistic effects with idelalisib in the SU-DHL-6 cell line.

This observation was also validated by the significantly higher inhibition of phosphorylated AKT (p-AKT), a representative marker of PI3K pathway activation, induced by co-exposure to JQ1 and idelalisib than exposure to other combinations (Figure 1B). Inhibition of the PI3K signaling by idelalisib was only transient because AKT phosphorylation rebounded over time, indicating PI3K pathway reactivation (Figure 1B). Consistent with our initial cell proliferation screening, adding a BRD4i (JQ1) strongly attenuated p-AKT reactivation in SU-DHL-6 cells, whereas other epigenetic drugs did not exhibit satisfactory inhibition (Figure 1B). The results of the two methods were consistent and the correlation analysis between the two screening methods showed that there was a positive correlation between the combination index and the inhibition rate of p-AKT, indicating that the effectiveness of the drug combination effectiveness was closely related to the continuous inhibition of the downstream of PI3K pathway (Figure 1C).

These findings were validated using eight cell lines originating from three main aggressive NHL types, DLBCL (SU-DHL-6, SU-DHL-4, and OCI-LY-3), MCL (JEKO-1 and Granta 519), and BL (namalva, CA-46, Raji, and Ramos). In addition, other hematological cell lines were investigated, including the acute myeloid leukemia MV4-11 cell line and two T-cell acute lymphoblastic leukemia cell lines CCRF-CEM and Jurkat cell lines, which reportedly express PI3Kδ. Consistently, a synergistic effect was observed with the combination of idelalisib and JQ1 in the eight tested cell lines derived from NHL while only additive to antagonistic effects were observed in the other hematological cell lines (Figure 1D, 1E).

Idelalisib and JQ1 mildly inhibited cell proliferation in assays of the three aggressive NHL cell lines. The half-maximal inhibitory concentration (IC50) values of idelalisib and JQ1 against SU-DHL-6, SU-DHL-4, and JEKO-1 cells were 117, 300, and 123 nM, respectively, and 59, 122, and 88 nM, respectively (Figure 1F). Additionally, cell viability assays showed strong synergistic effects with the combination of idelalisib and JQ1 in SU-DHL-6, SU-DHL-4, and JEKO-1 cells treated for 5 days (Figure 1G, 1H, all median confidence intervals [CIs]<0.5). However, this drug combination did not show strong
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

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F.
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

Figure 1. A. A total of 14 well-characterized compounds targeting seven classes of epigenetic regulators were tested in a combination screening with the PI3Kδi idelalisib, in DLBCL SU-DHL-6 cell. The CI values are described as synergism (CI<1), additive effect (CI=1), and antagonism (CI>1). The color intensity showed the mean of CI (blue, synergism; red, antagonism). B. Western blotting analysis of p-AKT expression after exposure to the seven classes of epigenetic regulators alone or in combination with idelalisib. C. The correlation analysis between the combination index and the inhibition rate of p-AKT. D. Mean CI values obtained in 12 lymphoma cell lines treated with different concentrations of idelalisib in combination with JQ1. Y-axis, CI (CI<0.8, synergism; 0.8<CI<1.2, additive effect; CI>1.2, antagonism). Results are expressed as mean ± SD. E. Mean CI values and 95% Confidence Intervals (C.I.) of the combination upon treatment with idelalisib in combination with JQ1. F. Dose-inhibition curves for SU-DHL-6, SU-DHL-4 and JEKO-1 cells treated with the indicated concentrations of idelalisib and JQ1 alone. Data represent average ± SD of three replicates. G. CI values for SU-DHL-6, SU-DHL-4 and JEKO-1 cells when treated with idelalisib in combination with JQ1 at the indicated concentrations. Mean CI values represents data from at least three independent experiments are shown. H. The combination effects (CI values) of two BRD4 inhibitors (JQ1 and OTX015) in combination with idelalisib in SU-DHL-6, SU-DHL-4, and JEKO-1 cells.
synergistic effects until after 72 h (Figure S2), probably because epigenetic regulators might take longer to exert anti-proliferative activity [20-22].

Furthermore, the synergistic anti-proliferative effects of BRD4 and PI3Kδ inhibition against the three aggressive NHL cell lines were further validated by combining other BRD4i (OTX15 and AZD5153) with idelalisib, indicating that the combined effect of idelalisib and a BRD4i was not specific to one agent (Figures 1H and S3A).

Combined PI3Kδ and BRD4 inhibition synergistically induced apoptosis of aggressive NHL cell lines

Both PI3Kδ and BRD4 protein inhibition obstruct cell proliferation by inducing apoptosis [12, 24]. Therefore, we speculated that combining a PI3Kδi and a BRD4i could enhance cancer cell apoptosis. We assessed induction of apoptosis after single or combined exposure to inhibitors in the three NHL cell lines using annexin V (AnnV) and propidium iodide (PI) co-staining followed by flow cytometry. Time-dependent apoptotic cell death was observed in SU-DHL-6, SU-DHL-4, and JEKO-1 cells following treatment with idelalisib and JQ1 alone, as well as in combination (Figure 2A, 2B).

Furthermore, apoptotic cell proportion in the co-treated group was significantly higher than that in either single inhibitor-treated group. Moreover, treatment of all three cell lines with idelalisib and JQ1 together for only 24 h significantly induced apoptosis, whereas either single drug exposure only slightly increased apoptosis (Figure 2A, 2B). These results suggest that the combination of idelalisib and JQ1 exerted synergistic anti-proliferative activity by enhancing and accelerating apoptosis.

We next examined the expression patterns of apoptosis-related proteins (poly-ADP ribose polymerase [PARP], B-cell lymphoma 2 [BCL2] family, and caspase 3) in these cell lines. Western blotting showed that idelalisib combined with JQ1 induced cleavage of PARP and caspase-3, two important indexes of cellular apoptosis, more potently than single treatment, which showed only a minimal effect (Figure 2C). Moreover, the combinatorial strategy also downregulated expression of BCL2 family proteins, including the anti-apoptotic BCL2 and BCL2-like 1 (BCL2L1, also known as BCL-X) proteins. In addition, the synergistical apoptosis inhibition of BRD4 and PI3Kδ in the aggressive NHL cell lines was further validated by combining another bivalent BRD4i (AZD5153), with idelalisib, indicating that the combined beneficial effect of idelalisib and BRD4i was not dependent on a specific BRD4i (Figures S3B, S3C).

Combined PI3Kδ and BRD4 Inhibition blocked PI3K signal reactivation in aggressive NHL cell lines

Previously, we found that reactivation of p-AKT in SU-DHL-6 cells after exposure to PI3Kδi was suppressed by BRD4i (Figure 1A). To elucidate the effects of BRD4i on PI3K pathway reactivation and of combining PI3Kδi and BRD4i in growth inhibition, we first treated all three cell lines with different concentrations (0.1-9 μM) of idelalisib or JQ1 and examined the effects on protein expression. Western blotting analysis indicated that idelalisib or JQ1 treatment alone caused a dose-dependent downregulation of p-AKT and c-MYC expression at the indicated times in all cell lines (Figure 3A).

We then treated all three cell lines with idelalisib and JQ1 alone or in combination and harvested cells at multiple time points (0, 2, 4, 8, 12, 24, and 48 h) from all three cell lines. We found that treatment with JQ1 downregulated p-AKT expression and the downstream pathway, as well as c-MYC, in a time-dependent manner in all cell lines (Figure 3B). However, idelalisib treatment only transiently blocked the PI3K signaling, as evidenced by the restoration of AKT, P70S6K, and 4E-BP1 phosphorylation 4-24 h later. The expression levels of these proteins recovered to the same or even higher levels than those of the control group after sustained idelalisib treatment (Figure 3C). Consistently, combining AZD5153 with idelalisib also synergistically suppressed the PI3K pathway consequently (Figure S3D).

Combined PI3Kδi and BRD4 inhibition simultaneously inhibited c-MYC transcription and translation

Previous studies have reported that c-MYC upregulation impairs responses to PI3Ks, which may be a potential mechanism of resistance to
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

A

SU-DHL-6

Control  |  Ideclalisib  |  JQ1  |  Comb

Annexin V

24h

48h

72h

PI

SU-DHL-4

Control  |  Ideclalisib  |  JQ1  |  Comb

Annexin V

24h

48h

72h

PI

JEKO-1

Control  |  Ideclalisib  |  JQ1  |  Comb

Annexin V

24h

48h

72h

PI

B

SU-DHL-6

Percentage of apoptotic cells (%)

Control  |  Ideclalisib  |  JQ1  |  Comb

24h

48h

72h

SU-DHL-4

Percentage of apoptotic cells (%)

Control  |  Ideclalisib  |  JQ1  |  Comb

24h

48h

72h
**Synergistic effects of BRD4i and PI3Ki on aggressive NHL**

Figure 2. Combined PI3Kδ and BRD4 inhibition induced synergistic apoptosis of aggressive NHL cell lines. A. FCM analysis of the degree of apoptosis in three cell lines after treatment with idelalisib alone or in combination with JQ1 for 24, 48, 72 h. For SU-DHL-6 and JEKO-1, idelalisib and JQ1 were at 1 μM; for SU-DHL-4, idelalisib and JQ1 were at 3 μM. B. Quantification of the FCM analysis was shown. *P<0.05, **P<0.01, ***P<0.001. The combined group vs idelalisib or JQ1 alone. Results are expressed as mean ± SD. C. Western blotting analysis of apoptosis cascade related protein expression levels, including cleaved PARP, BCL2, BCL2L1 and cleaved caspase-3, after treated by idelalisib and JQ1 alone or in combination for 72 h.
Figure 3. Combined PI3Kδ and BRD4 inhibition blocked PI3K signal reactivation in aggressive NHL cell lines. A. Western blotting analysis for p-AKT, AKT and c-MYC after treatment with idelalisib for 24 h or JQ1 for 48 h. For SU-DHL-6 and JEKO-1, idelalisib and JQ1 were at 1 μM; for SU-DHL-4, idelalisib and JQ1 were at 3 μM. B. SU-DHL-6, SU-DHL-4 and JEKO-1 cells were treated with JQ1 for the indicated time points, and the expression levels of p-AKT, AKT, p-P70S6K, P70S6K, p-4EBP1, 4EBP1, and c-MYC were assessed by western blotting. For SU-DHL-6 and JEKO-1, JQ1 was at 1 μM; for SU-DHL-4, JQ1 was at 1 μM. C. Western blotting analysis in SU-DHL-6, SU-DHL-4 and JEKO-1 cells treated with idelalisib with or without JQ1 for the indicated time points. For SU-DHL-6 and JEKO-1, idelalisib and JQ1 were at 1 μM; for SU-DHL-4, idelalisib and JQ1 were at 3 μM.
this class of drugs with direct clinical implications [17]. We have demonstrated that combining a PI3Kδi (idelalisib) with a BRD4i (JQ1) suppressed c-MYC protein expression in DLBCL and MCL cell lines. Next, we sought to examine the mechanism of action of this drug combination on c-MYC by analyzing transcription and translation. Exposure to idelalisib or JQ1 alone reduced c-MYC protein levels in a concentration dependent manner, whereas co-treatment resulted in a more significant reduction (Figure 3A-C).

Furthermore, expression of c-MYC was not restored by a potent proteasome inhibitor, MG-132, implying that idelalisib and JQ1 alone or in combination could not induce degradation of c-MYC (Figure 4A). Studies have demonstrated that BRD4 inhibition downregulates c-MYC expression by inhibiting transcription [27, 28]. Our subsequent quantitative real time-PCR (qRT-PCR) analysis showed that JQ1 alone, but not idelalisib, substantially reduced the transcription of c-MYC (Figure 4B). Furthermore, adding idelalisib also did not increase the inhibition of transcription by JQ1, suggesting that the enhanced inhibition of c-MYC expression by co-treatment with idelalisib and JQ1 could not synergistically suppress transcription of c-MYC (Figure 4B).

Previous studies have reported that idelalisib stimulated the mTOR pathway through AKT and further activated P70S6K and 4EBP-1, activating target translation in DLBCL and MCL cells [29, 30]. To further confirm the effect of combining these two drugs on c-MYC translation, we performed western blots on the three cell lines against two well-known translation-related targets of the PI3K pathway, eukaryotic translation initiation factor 4B (eIF4B) and eIF4E. As shown in Figure 4C, eIF4B and eIF4E proteins were significantly reduced in the combination groups of all cell lines, confirming that co-treatment with idelalisib and JQ1 suppressed translation of c-MYC (Figure 4B). Together, these results demonstrate that combined PI3Kδ and BRD4 inhibition served to control the expression of c-MYC protein by specifically silencing its transcription and translation, increasing the response to PI3Kδi (Figure 4D).

Combined PI3Kδ and BRD4 inhibition suppressed tumor growth of aggressive NHL in vivo

The combination of idelalisib and JQ1 exhibited high synergistic activity in vitro. Therefore, we assessed the efficacy of the compounds alone or in combination in reducing tumor growth of two in vivo lymphoma models: SU-DHL-6 and JEKO-1 cells engrafted into NOD-SCID mice (Figure 5A). Strikingly, the idelalisib and JQ1 combination greatly reduced tumor growth in both models more than either compound alone (Figure 5A, 5B). In the SU-DHL-6 model, idelalisib and JQ1 exhibited tumor growth inhibition (TGI) of 48.76% and 54.15%, respectively, whereas co-treatment showed near stasis with a TGI of 70.64%. In the JEKO-1 model, idelalisib and JQ1 alone or in combination resulted in TGI of 48.86%, 59.20%, and 78.00%, respectively, implying that the observed synergistic in vitro anti-proliferative activity was reproduced in vivo. In addition, both mono- and co-treatments were well tolerated by the mice with no body weight loss was detected (Figure 5C).

To validate the in vivo assay results, we evaluated the expression of PI3K pathway proteins, p-AKT, p-4EBP1, p-P70S6K, and c-MYC and the apoptosis marker, cleaved caspase 3, using immunohistochemical (IHC) analyses of tumor samples from the established mouse models. The p-AKT-, p-4EBP1-, p-P70S6K-, and c-MYC-positive areas of the IHC staining decreased more markedly in the idelalisib and JQ1 combination group than in the single agent groups, whereas more cleaved caspase 3-positive cells were observed (Figure 5D, 5E). Cumulatively, these findings suggest that combining PI3Kδi and BRD4 inhibition suppressed the growth of aggressive NHL in vivo.

Combined PI3Kδ and BRD4 inhibition suppressed aggressive NHL after drug withdrawal

Based on the high synergistic effect of with idelalisib and JQ1 co-treatment, specifically resulting in inhibition of the PI3K pathway and c-MYC, we evaluated the potential residual anti-proliferative effects after drug withdrawal. Cell counting assays indicated that 24 h after withdrawal of idelalisib or JQ1 alone, cancer cells
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

A

SU-DHL-6
C Idealisib JQ1 comb
MG-132 - + + +
c-MYC
GAPDH

SU-DHL-4
C Idealisib JQ1 comb
MG-132 - + + +
c-MYC
GAPDH

JEKO-1
C Idealisib JQ1 comb
MG-132 - + + +
c-MYC
GAPDH

B

SU-DHL-6-c-MYC
Normalized Fold Expression
Control Idelisib JQ1 Comb

SU-DHL-4-c-MYC
Normalized Fold Expression
Control Idelisib JQ1 Comb

JEKO-1-c-MYC
Normalized Fold Expression
Control Idelisib JQ1 Comb

C

SU-DHL-6
C Idealisib JQ1 comb
eIF4B
eIF4E
GAPDH

SU-DHL-4
C Idealisib JQ1 comb
eIF4B
eIF4E
GAPDH

JEKO-1
C Idealisib JQ1 comb
eIF4B
eIF4E
GAPDH

55 kDa
36 kDa
80 kDa
25 kDa
36 kDa
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

**Figure 4.** Combined PI3Kδi and BRD4i inhibition simultaneously inhibited c-MYC transcription and translation. A. Western blotting analysis for c-MYC in idelalisib and JQ1 treatment alone or in combination after addition of MG-132 (10 μM). B. Expression levels of c-MYC mRNA in SU-DHL-6, SU-DHL-4 and JEKO-1 cells treated as indicated for 24 h. For SU-DHL6 and JEKO-1, idelalisib and JQ1 were at 1 μM; for SU-DHL-4, idelalisib and JQ1 were at 3 μM. *P<0.05, **P<0.01, ***P<0.001. The combined group vs idelalisib or JQ1 alone. Results are expressed as mean ± SD. C. Western blotting analysis for two translation-related targets of PI3Ks pathway, namely elf4B and elf4E after treatment with idelalisib and JQ1 alone or in combination for 48 h. D. A schematic model of the mechanism of PI3Kδi and BRD4i inhibition in c-MYC and results in effects c-MYC transcription and translation program.
Synergistic effects of BRD4i and PI3Ki on aggressive NHL
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

Figure 5. Combination of PI3Kδ and BRD4 inhibition suppressed tumor growth of aggressive NHL in vivo. In-vivo efficacy of idelalisib and JQ1 alone or in combination in SU-DHL-6 and JEKO-1 xenograft models (n = 5). NOD-SCID mice were orally treated with vehicle control, idelalisib (25 mg/Kg), JQ1 (50 mg/Kg) or in combination once every day for indicated days. A. Volume of tumors from sacrificed mice are shown, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Mean tumor volumes ± SD. B. Weight of tumors from sacrificed mice were shown, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (Horizontal lines represent the means ± SD). C. Data showing average body weight changes of the vehicle, idelalisib, JQ1 and the combined group treated mice in xenograft model. Points, mean value; bars, SD. D. Tumor tissues from SU-DHL-6 and JEKO-1 xenograft treated with vehicle control, idelalisib, JQ1 and the combined group for indicated days were immunohistochemically analyzed with anti-p-AKT, anti-p-P70S6K, anti-p-4EBP1, c-MYC and anti-cleaved caspase-3 (n = 4). Representative images were shown. Scale bar = 50 μm. E. Quantification of percentage of positive staining was shown. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. The combined group vs idelalisib or JQ1 alone. Horizontal lines represent the means ± SD.
survived and proliferated continuously for up to 72 h. In contrast, the combination group cells did not proliferate after prolonged withdrawal (Figure 6A). These results demonstrate that the combination of idelalisib and JQ1 continued to inhibit the growth of SU-DHL-6 and JEKO-1 cancer cells after withdrawal.

Consistent with the results of the anti-proliferative assay, the levels of AKT, P70S6K, 4E-BP1, and c-MYC phosphorylation in the combination group were persistently downregulated following drug withdrawal, while in the single drug groups the phosphorylation of PI3K downstream components were readily restored after drug withdrawal (Figure 6B). These results suggested that the combination strategy may achieve superior anti-tumor activity in vivo compared to single drug application.

We next assessed the in vivo residual anti-tumor efficacy of the combination strategy after drug withdrawal in two lymphoma models. In particular, rats treated with idelalisib (25 mg/kg) or JQ1 (50 mg/kg) alone exhibited rapid tumor regrowth 12 and 9 days after drug withdrawal in the SU-DHL-6 and JEKO-1 cell models, respectively. Contrastingly, the combination group showed a durable anti-tumor response even after drug withdrawal in vivo, with only a slight increase in tumor volume (Figure 6C). Furthermore, the p-AKT-, p-4EBP1-, p-P70S6K-, and c-MYC-positive area of the IHC staining in the co-treated group also consistently decreased (Figure 6D, 6E).

Discussion

BCR signaling and the downstream PI3K pathway play several important roles in lymphoid tumors derived from mature B-cells and contain various potential therapeutic targets [2-4]. PI3Kδ is highly expressed, primarily in the B-cell hematopoietic system, and is crucial for B-cell survival. Currently, targeting PI3Kδ has been one of the most successful strategies for treatment of iNHL and CLL [9-11]. However, patients with aggressive NHLs, such as DLBCLs and MCLs, have fewer therapeutic options as they do not experience significant clinical benefit from PI3Kδ inhibitors.

In the current study we have demonstrated that idelalisib failed to continuously suppress PI3K signaling in aggressive NHL cell lines (Figure 3C), demonstrating that new therapeutic strategies are needed to for sensitize aggressive NHLs to PI3Kδi treatment. Drug combinations can effectively enhance anticancer drug efficacy and reduce the emergence of drug resistance. We, therefore, screened epigenetic drugs to identify possible candidates that could be combined with idelalisib to prevent PI3K pathway reactivation after treatment and inhibit the proliferation of aggressive NHL cell lines.

Activity analysis of the 14 compounds against seven epigenetic targets led to the identification of two BRD4 inhibitors, JQ1 and OTX015, that exhibited the most significant effect on downregulating p-AKT and showed strong synergistic anti-proliferative effects in combination with idelalisib in a panel of aggressive NHL cell lines. Moreover, the synergistic anticancer effects were further reproduced in several DLBCL (SU-DHL-6, SU-DHL-4) and MCL (JEKO-1) cell lines as well as two xenograft models. More importantly, the synergistic anticancer effects persisted both in vitro and in vivo even after prolonged drug withdrawal, demonstrating the effectiveness of co-treatment with PI3Kδi and BRD4i. Further investigations revealed that the synergistic anti-tumor effects of co-treatment with PI3Kδi and BRD4i could be attributed to (i) BRD4i enhancing the inhibitory activity of idelalisib on the PI3K pathway, which further induced the expression of apoptotic proteins and inhibited expression of anti-apoptotic proteins thereby enhancing apoptosis; (ii) co-treatment with BRD4i and idelalisib simultaneously inhibited transcription and translation of c-MYC, which synergistically downregulated c-MYC expression; (iii) sustained downregulation of the PI3K pathway and c-MYC protein expression induced by the combination strategy continuously suppressing cell proliferation and tumor growth in vivo over a longer period than that induced by any single drug.

A recent study has also reported that BRD4 inhibition blocked feedback activation of numerous tyrosine kinase receptors (RTKs), which could activate PI3K signaling pathways. Meanwhile, combining a pan-class I PI3Ki and BRD4i sustained PI3K pathway inhibition and enhanced tumor cell death [16]. Inhibition of BRD4 also inhibited nuclear factor (NF)-κB and attenuated expression of several NF-κB-activated pro-growth and pro-survival genes,
Synergistic effects of BRD4i and PI3Ki on aggressive NHL
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

Figure 6. Combined PI3Kδ and BRD4 inhibition suppressed aggressive NHL after drug withdrawal. A. The quantifications of live cells after drug withdraw of the indicated groups were in the bottom panel. *P<0.05, **P<0.01, ***P<0.001 vs base line (zero). B. Western blotting analysis of p-AKT, AKT, p-P70S6K, P70S6K, p-4EBP1, 4EBP1 and c-MYC after drug withdraw of idelalisib and JQ1 alone or combined group for the indicated time points. C. In-vivo efficacy after drug withdraw as indicated group were shown. Mean tumor volumes ± SD of tumors from sacrificed mice (Horizontal lines represent the means ± SD), *P<0.05, **P <0.01, ***P<0.001, ****P<0.0001. D. Tumor tissues from SU-DHL-6 and JEKO-1 xenograft of drug withdraw as indicated group were immunohistochemically analyzed with anti-p-AKT, anti-p-P70S6K, anti-p-4EBP1, c-MYC (n = 4). Representative images were shown. Scale bar = 50 μm. E. Quantification of percentage of positive staining was shown. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. The combined group vs idelalisib or JQ1 alone. Horizontal lines represent the means ± SD.
including BTK, in MCL cells [26]. These effects could indirectly inhibit activation of the PI3K pathway and rationally explain our observation that BRD4i inhibited feedback activation of the PI3K pathway after idelalisib treatment.

Moreover, previous studies have reported that BETi enhanced the susceptibility of the ABC-DLBCL and BL cell line-derived lymphomas to PI3Ki. This synergistic effect depends on glycogen synthase kinase 3β (GSK3β) feedback induced by BRD4i, which is a result of the consequential increase of p-GSK3β S9 expression levels [18]. In GCB-DLBCL and MCL cell lines, BRD4i had no significant effect on p-GSK3β S9 expression levels, however, persistently inhibited the PI3K downstream pathway in combination with the selective PI3Kδi. We speculated that the inhibitory feedback regulation of GSK3β was not the primary mechanism mediating the synergistic anti-proliferative effect of PI3Kδi and BRD4i on GCB-DLBCL and MCL cell lines.

c-MYC is one of the most frequently altered genes in aggressive NHL cell lines, and is thus an attractive therapeutic target. However, there are currently no known direct inhibitors of c-MYC [27]. Upregulation of c-MYC could be an underlying mechanism impairing responses to PI3Ki (Figure 3C) and co-treatment with idelalisib and JQ1 could markedly decrease c-MYC expression in DLBCL and MCL cell lines. The addition of MG-132 did not recover the expression of c-MYC, and the combination of idelalisib and JQ1 also did not decrease the transcription of c-MYC when compared to JQ1 alone. Therefore, we believe that idelalisib alone or in combination with JQ1, could inhibit the translation of c-MYC.

c-MYC is reported to be a downstream target of the PI3K/AKT pathway [29]. Furthermore, PI3K/AKT/mTOR-dependent phosphorylation of 4EBP1 and P70S6K positively regulates the elf4 complex, thus promoting the translation of mRNAs containing long 5’-untranslated regions (5’-UTRs) with complex RNA secondary structures, such as c-MYC [29]. Correspondingly, the combination of idelalisib and JQ1 inhibited 4EBP1 and P70S6K phosphorylation and subsequently decreased elf4B and elf4E, thereby repressing c-MYC translation. However, the mechanism underlying the inhibition of elf4B and elf4E in cells treated with idelalisib and JQ1, as well as the relationship of that inhibition to c-MYC downregulation require further study.

Sustained inhibition of cancer cell proliferation and tumor growth are crucial for clinical efficacy, but there are few available methods to evaluate the duration of anticancer effects after drug withdrawal. Cancer cell proliferation and tumor growth rates after drug withdrawal were significantly lower in the combination treatment than any single drug, indicating the therapeutic value of this drug combination. The durable anticancer effect may be mediated by persistent inhibition of the PI3K pathway and expression of c-MYC. Although the mechanism underlying this persistent anticancer effect was not fully elucidated, our study provides a reliable pharmacodynamic method to evaluate the advantages of drug combinations over single drug use.

In conclusion, PI3Kδ and BRD4 inhibition can be effectively combined to achieve comprehensive and durable suppression of the PI3K/AKT/mTOR pathway, resulting in synergistic antitumor activity against a broad range of aggressive NHL cell models, especially in DLBCL and MCL. This study provides a potential effective treatment strategy for aggressive NHL. Further studies on primary cancer cell lines would establish a stronger rationale for its clinical application.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

PI3Ks, Phosphatidylinositol 3-kinases; NHL, Non-Hodgkin lymphomas; PI3Kδi, PI3Kδ inhibitors; BRD4i, BRD4 inhibitors; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphomas; MCL, mantle cell lymphomas; BL, Burkitt lymphomas; DMSO, Dimethyl Sulfoxide; BCL2, B-cell lymphoma 2; BCL2L1, B-cell lymphoma 2-like 1; TGIs, tumor growth inhibitors.
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

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References


Synergistic effects of BRD4i and PI3Ki on aggressive NHL


Synergistic effects of BRD4i and PI3Ki on aggressive NHL

**Table S1.** The list of antibodies and related information in this article

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**Table S2.** The primer sequences used in qRT-PCR

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Synergistic effects of BRD4i and PI3Ki on aggressive NHL
Figure S1. Drug combination screening identifies BRD4i as acting synergistically with PI3Kδi. A total of 14 well-characterized compounds targeting seven classes of epigenetic regulators were tested in a combination screening with the PI3Kδi idelalisib, in DLBCL SU-DHL-6 cell. Mean CI values represents data from at least three independent experiments are shown.
Synergistic effects of BRD4i and PI3Ki on aggressive NHL

Figure S2. The effect of combining PI3Kδ and BRD4 inhibition was time dependence. Percentage inhibition for SU-DHL-6 and JEKO-1 cells when treated with idelalisib in combination with JQ1 at the indicated time is presented.
Figure S3. The effect of combining PI3Kδi idelalisib and BRD4i AZD5153 in vitro. A. CI values for SU-DHL-6 and JEKO-1 cells when treated with idelalisib in combination with AZD5153 at the indicated concentrations. Mean CI values represents data from at least three independent experiments are shown. B. FCM analysis of the degree of apoptosis in three cell lines after treatment with idelalisib (1 μM) alone or in combination with AZD5153 (100 nM) for 72 h. Quantification of the FCM analysis was shown. *P<0.05, **P<0.01, ***P<0.001. The combined group vs idelalisib or AZD5153 alone. Results are expressed as mean ± SD. C. Western blotting analysis of apoptosis cascade related protein expression levels, including cleaved PARP, BCL2, BCL2L1 and cleaved caspase-3, after treated by idelalisib (1 μM) and AZD5153 (100 nM) alone or in combination for 72 h. D. Western blotting analysis in SU-DHL-6 and JEKO-1 cells treated with idelalisib (1 μM) alone or in combination with AZD5153 (100 nM) for 48 h.