Am J Cancer Res 2023;13(2):452-463
www.ajcr.us /ISSN:2156-6976/ajcr0148314

Original Article

A combination of BR101801 and venetoclax enhances antitumor effect in DLBCL cells via c-Myc/Bcl-2/Mcl-1 triple targeting

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Received December 7, 2022; Accepted January 12, 2023; Epub February 15, 2023; Published February 28, 2023

Abstract: Double hit diffuse large B-cell lymphoma (DLBCL) with rearrangement and overexpression of both c-Myc and Bcl-2 responds poorly to standard R-CHOP therapy. In a recent phase I study, Venetoclax (ABT-199) targeting Bcl-2 also exhibited disappointing response rates in patients with relapsed/refractory DLBCL, suggesting that targeting only Bcl-2 is not sufficient for achieving successful efficacy due to the concurrent oncogenic function of c-Myc expression and drug resistance following an increase in Mcl-1. Therefore, co-targeting c-Myc and Mcl-1 could be a key combinatorial strategy to enhance the efficacy of Venetoclax. In this study, BR101801 a novel drug for DLBCL, effectively inhibited DLBCL cell growth/proliferation, induced cell cycle arrest, and markedly inhibited G0/G1 arrest. The apoptotic effect of BR101801 was also observed by increased Cytochrome C, cleaved PARP, and Annexin V-positive cell populations. This anti-cancer effect of BR101801 was confirmed in animal models, where it effectively inhibited tumor growth by reducing the expression of both c-Myc and Mcl-1. Furthermore, BR101801 exhibited a significant synergistic antitumor effect even in late xenograft models when combined with Venetoclax. Our data strongly suggest that c-Myc/Bcl-2/Mcl-1 triple targeting through a combination of BR101801 and Venetoclax could be a potential clinical option for double-hit DLBCL.

Keywords: Double hit diffuse large B-cell lymphoma, c-Myc, Bcl-2, Mcl-1, BR101801, Venetoclax

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL) of B-cell origin and is characterized by rapid progression and heterogeneity in the diagnostic category [1]. Most DLBCL patients respond to rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) therapy. However, 40% of DLBCL patients face relapsed or refractory disease, and most of them die of lymphoma [2]. Double-hit DLBCL is a highly aggressive subtype of DLBCL in which both Myc and Bcl-2 are rearranged and overexpressed [3]. A double-hit population is associated with poor prognosis and residency on R-CHOP therapy, and those with double-hit DLBCL experience relapses within a shorter time [4]. Therefore, there is an unmet need to identify novel therapeutic approaches and drug combinations for patients with double-hit DLBCL.

The phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) pathway is known to be closely involved in cancer progression in various types of cancer [5], and PI3K inhibitors have already been tested for clinical use either as a single agent or in combination with other agents for cancer treatment [6-8]. Activated PI3K is detectable in a significant number of DLBCL patient samples [9], and several recent studies have suggested that the PI3K signaling pathway is an attractive therapeutic target for DLBCL patients [10-12]. Indeed, suppression of PI3K induces apoptosis and cell cycle arrest in DLBCL by inhibiting multiple pathways, including JAK/STAT3, MAPK/ERK, and NF-kB [8].

Importantly, the PI3K pathway is a major upstream signaling pathway regulating c-Myc expression, and its inhibition suppresses DLBCL tumor growth [13].

Venetoclax (ABT-199, GDC-0199) is the first approved BCL-2 inhibitor that has been widely used in the treatment of chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) [14]. Unfortunately, Venetoclax has shown an unsatisfactory response rate [CR: 4/34 (12%), PR: 2/34 (6%)] in a recent phase I study of patients with relapsed/refractory DLBCL [15]. The cause of the failure of Venetoclax in DLBCL is thought to be that it does not cover the oncogenic activity at high c-Myc levels and Bcl-2 bypasses anti-apoptotic pathways such as Mcl-1 [16]. In this regard, a recent study targeting both Bcl2 and PI3K/c-Myc showed improved apoptosis-dependent antitumor effects [17, 18], implying that multi-targeting, including that of c-Myc and Bcl-2, may be a game-changer for DLBCL treatment. In our previous study, we developed BR101801 as an oral drug targeting PI3K and recently reported that it could function as a dual inhibitor of DNA-PK and PI3K^{TM}, and as an immunomodulatory radiosensitizer in solid tumor models [19, 20]. In the current study, we showed that BR101801 can suppress c-Myc and Mcl-1 and inhibit the PI3K signaling pathway and that c-Myc/Mcl-1/Bcl-2 triple targeting via the combination of BR101801 and Venetoclax is a potential therapeutic option for double-hit DLBCL.

Materials and methods

Preparation of BR101801

The reaction of 1-(4, 6-dichloropyrimidin-5-yl) ethan-1-one (1) with 4-methoxybenzylamine (PMBA) generated aminopyrimidine under a base. This was followed by ring closing with DMF-DMA to give compound (2). The amine of (S)-3-(1-aminoethyl)-8-chloro-2-phenylisoquinolin-1(2H)-one (3) was protected with trifluoroacetic anhydride to give compound (4). Compound (4) was reacted with N-chlorosuccinimide (NCS), followed by NCS chlorination to give compound (5). Compound (5) was reacted with an aqueous solution of the base to give compound (6). Nucleophilic substitution with compound (2) and deprotection gave BR101801 (**Figure 1**).

Compounds

Venetoclax (ABT-199, A8194) was purchased from APExBIO, and Idelalisib (S2226), Ibrutinib (S2680), NU7441 (S2638), cycloheximide (S7418), and MG-132 (S2619) were purchased from Selleck Chemicals. The compounds were dissolved in DMSO and stored at -20°C until use. All compounds were added to the cells such that the final concentration of DMSO was less than 0.1%, and the results were compared with those obtained with controls incubated with 0.1% DMSO alone.

Cell culture

All DLBCL cell lines including SU-DHL-6 (diffuse large B cell lymphoma DLBCL GCB, DSMZ) and DOHH-2 (follicular lymphoma FL, DSMZ), were maintained in RPMI 1640 medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

PI3K isoform in vitro kinase assay

The enzyme selectivity of BR101801 was determined by Eurofins kinase profiling using homogenous time resolved fluorescence (HTRF) assays.

PI3K isoform cell based assay

The cellular mechanistic IC50 values were assessed by measuring phospho-AKT (Ser473) levels driven by different PI3K isoforms. To determine the activity of PI3K alpha, beta, delta, and gamma isoforms, NIH3T3, NIH3T3, Raji, and Raw264.7 cells were seeded in 6-well plates and starved for 24 h. The cells were then treated with BR101801 for 1 h, followed by treatment with 20 ng/mL PDGF (Sigma-Aldrich, Saint Louis, MO, USA, SRP4988) for 10 min, 10 μM LPA for 10 min, 0.25 μg/mL IgM (eBioscience, San Diego, CA, USA,14-9998-82) for 10 min and 10 ng/mL C5a (R&D systems, 2037-C5-025) for 5 min. The cells were lysed and Akt phosphorylation was determined by ELISA. The OD values were quantified at 450 nm using a microplate reader. The IC50 values were calculated by the GraphPad Prism software.

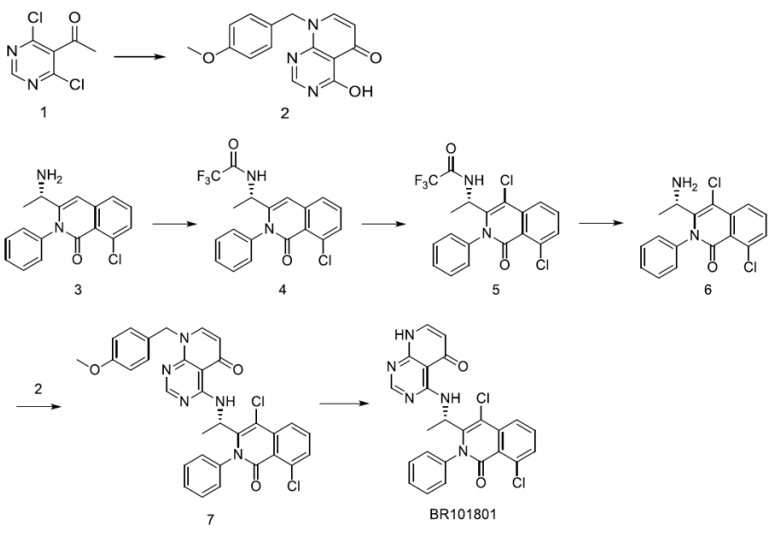

Figure 1. Synthetic process and structure of BR101801.

Cell cycle analysis

The cells were treated with various concentrations of BR101801 for 24 h and then centrifuged at 1000 rpm at 4°C. They were then washed with PBS and fixed with 70% cold ethanol. After overnight incubation at -20°C, the cells were washed with PBS and resuspended in a PI/RNase Staining Buffer (BD Biosciences, San Jose, CA, USA, 550825) for 20 min at room temperature. DNA content was determined using Becton Dickinson (BD) FACSVerse™ flow cytometry analyzers, and the cell cycle profile was analyzed using ModFit LT software (Verity Software House).

Western blotting

Protein lysates were prepared using the RIPA lysis buffer (Sigma-Aldrich, R0278) supplemented with a protease/phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA, 1861281). The protein lysates were mixed with loading dye (Bio-Rad, 161-0747) and then equivalent amounts of protein were loaded per lane. Protein separation was achieved with electrophoresis using 4-12% Bis-Tris gel (Thermo Scientific, NP0321), and then the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Protein transfer was assessed using Ponceau S solution staining (Biosesang, Korea, PR2059-050-00). The membranes were blocked with phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 h and incubated overnight at 4°C with anti-caspase3 (Santa Cruz Biotechnology, Dallas, TX, USA, 71-48), anti-Cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA, 9661), anti-PARP (Cell Signaling Technology, 9542), anti-Mcl-1 (Cell Signaling Technology, 5453S), anti-c-Myc (Cell Signaling Technology, 5605S), and anti-β-actin (Cell Signaling Technology, 4967S) antibodies. After being washed three times with PBST, the membrane was incubated with a secondary antibody for 1 h. The secondary antibodies were diluted to 1:2000 in 5% skim milk. The proteins were visualized using ClarityTM Western ECL Substrate (Amersham Biosciences, Piscataway, NJ, USA).

Annexin V assay

DLBCL cell lines were seeded in 100-mm culture dishes at an approximately 70% confluence and incubated at 37°C for 24 h. The next day, cells were treated with BR101801 for 12 h. The medium from the attached cells was removed, and the cells were briefly washed twice with cold DPBS, double-stained with Annexin V (APC) and PI (BD Biosciences) in Annexin V binding buffer and analyzed on a FACS Calibur flow cytometer (Beckman Coulter, Indianapolis, IN, USA) equipped with a 488 nm argon laser. Live cells were gated using forward and side scatter to avoid non-specific fluorescence from dead cells.

Cytochrome C staining assay

DLBCL cells were seeded on 18-mm glass coverslips and grown to approximately 70% confluence. The cells were treated with BR101801 for 16 h. The cells were washed twice with DPBS, fixed in acetic acid: ethanol (1:2 v/v) solution for 10 min at -20°C, and incubated overnight at 4°C with an anti-Cytochrome C antibody (1:50 dilution, Santa Cruz Biotechnologies). The following day, the cells were washed twice with DPBS and incubated with a fluorescently labeled anti-mouse secondary antibody (1:100 dilution; Dianova, Hamburg,

Germany) for 1 h at room temperature. The cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Subsequently, the slides were washed twice with DPBS, mounted with 1, 4-diazabicyclo [2.2.2] octane (DABCO) (Sigma-Aldrich), and viewed under a confocal laser-scanning microscope (Fluo View 1000; Olympus).

Subcutaneous xenograft models

Female CB17 SCID mice (Charles River) were 5-6 weeks old on day 1 of the study and had a body weight range of 18-20 g. The animals had free access to sterile water and Lab Diet® throughout the study period. DOHH-2 and SU-DHL-6 cells were sub-cultured twice weekly. Cells growing in the exponential growth phase were harvested and counted for tumor inoculation. Each mouse was inoculated subcutaneously in the abdominal region with DOHH-2 and SU-DHL-6 cells (5×10^6) in 200 µL of PBS mixed with Matrigel (1:1) for tumor development. After cell inoculation, the animals were checked daily for morbidity and mortality. Tumor volumes were measured thrice weekly in two dimensions using a caliper, and the volume was expressed in mm^3 using the formula: V = 0.5 (a) × (b) 2, where (a) and (b) are the long and short diameters of the tumor, respectively. The tumor weight was measured at the time of termination of the study. BR101801, Ibrutinib, and Venetoclax were orally administered daily. The compounds were formulated as follows. Dosing solutions of BR101801 at 5 mg/mL, ibrutinib at 3 mg/mL and Venetoclax at 10 mg/mL were prepared in a vehicle (5% DMSO, 55% PEG400, 40% DI water), which provided active doses of 50, 30, and 100 mg/kg in a dosing volume of 10 mL/kg (0.2 mL/20 g mouse). The concentrations of the solutions were adjusted for body weight. The treatments were initiated when the mean tumor size reached an average volume of 80-120 mm^3 or 500-600 mm^3 (the model for late stage).

Immunohistochemistry

Immunostaining was performed using 5-µm-thick sections of tumor samples after deparaffinization. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 30 min, followed by permeabilization with 0.5% Triton ×-100 in PBS for 10 min. Peroxidase quenching was performed using 0.3% hydrogen peroxide (H_2O_2) in PBS for 10 min; then, the samples were pre-blocked with CAS block solution (Life Technologies) for 1 h at room temperature. They were then incubated overnight with the primary antibodies anti-Mcl-1 (Cell Signaling Technology, 5453S), anti-c-Myc (Cell Signaling Technology, 5605S), anti-Cleaved caspase3 (Cell Signaling Technology, 9661) at 4°C. For DAB staining, the sections were incubated for 1 h with biotinylated secondary antibodies (1:60) and streptavidin-HRP was applied. The sections were developed using diaminobenzidine tetrahydrochloride substrate, and counterstained with hematoxylin. For immunofluorescence, the sections were incubated for 1 h with fluorescently labeled secondary antibodies (1:60) and counterstained with 4, 6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. At least three random fields were examined in each section at × 200 magnification.

Statistical analysis

Statistical calculations were performed using the SPSS software for Windows (version 10.0, SPSS, Chicago, IL, USA). Results are expressed as mean ± standard deviation (SD) and were considered statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

BR101801, a novel PI3K inhibitor, effectively inhibits the proliferation of various DLBCL cell lines with superior efficacy than idelalisib or TGR-1202

To determine the enzyme selectivity of BR101801, we performed kinase profiling using homogenous time-resolved fluorescence (HTRF) assays. As shown in **Figure 2A**, BR101801 inhibited the activity of all PI3K subtypes at the nanomolar level and showed significant inhibitory effects, particularly on PI3Kγ and ™, resulting in lower IC50 values of 15 and 2, respectively. To investigate the anti-cancer effects of BR101801 on DLBCL, cell growth assays were performed using DLBCL cell lines that had been treated with various concentrations of BR101801 along with FDA-approved PI3K inhibitors (positive controls: Idelalisib or TGR-1202). The results revealed that BR101801 effectively inhibited the growth of most DLBCL cell lines and exhibited more sensitive GI50 values than Idelalisib or TGR-1202 (**Figure**

A **Enzymatic potency (cell free)**

Isotypes	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ
BR101801 IC50 (nM)	106	171	15	2

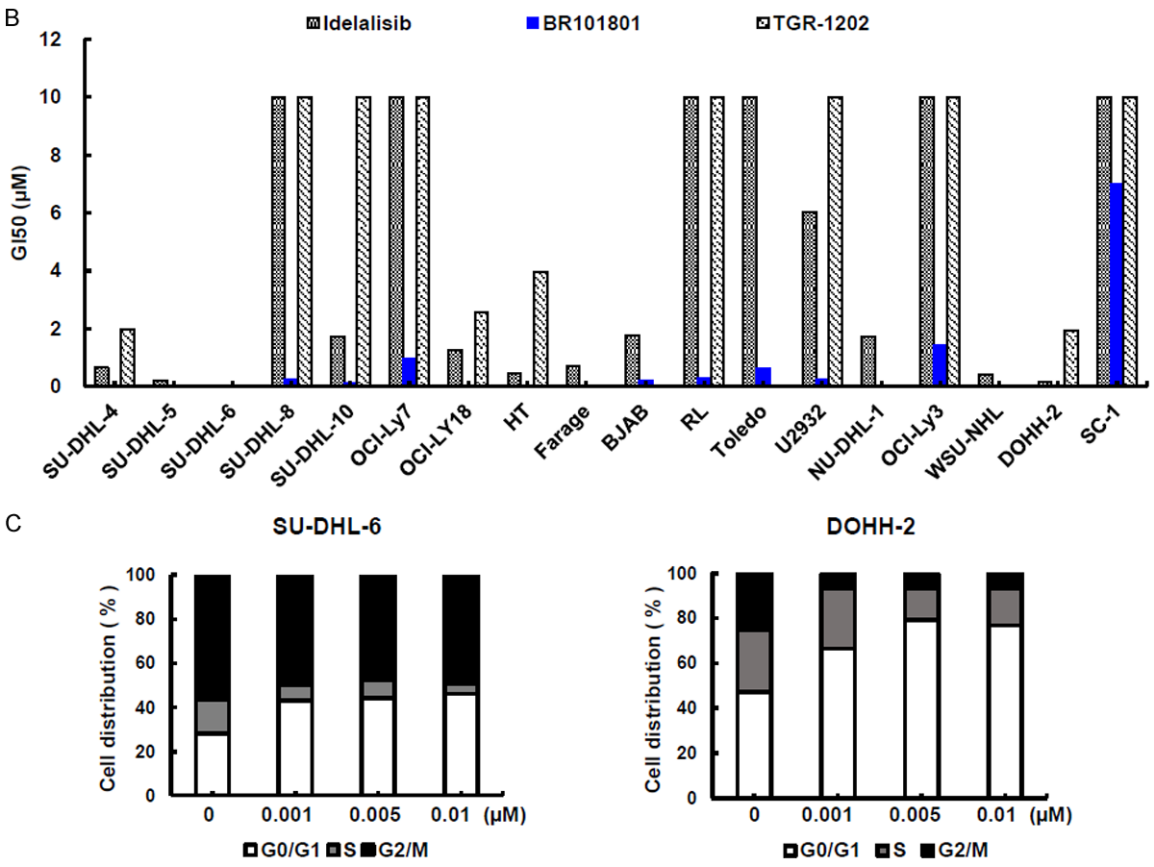

Figure 2. BR101801, a novel PI3K inhibitor, effectively inhibits proliferation of various DLBCL cell lines. A. In vitro selectivity of BR1010801 on different PI3K subtypes was studied in cell-free systems using Homogenous Time Resolved Fluorescence (HTRF) analysis. B. Growth inhibition (GI50) of DLBCL cells by BR101801. The in vitro anti-cancer effects of BR101801 were confirmed in various cell lines. C. Flow cytometry was performed to detect cell cycle distribution. After treatment with BR101801 (0-0.01 μM) for 16 h, the DLBCL cell lines were subjected to a propidium iodide (PI) staining assay.

2B). Moreover, when we analyzed the cell cycle distribution of SU-DHL-6 and DOHH-2 cells after BR101801 treatment, we found that this treatment increased the G0/G1 phase cell population in a dose-dependent manner (**Figure 2C**). These data reveal that the novel PI3K inhibitor BR101801 effectively inhibits the growth of DLBCL cells.

BR101801 exhibits an anti-cancer effect by inducing apoptosis of DLBCL

BR101801 treatment induces apoptosis and cell cycle arrest in DLBCL cell lines. BR101801 induced the Cleavage of caspase 3 (the active form of caspase 3), which led to PARP cleavage (**Figure 3A**) in a dose-dependent manner. When we treated the cell lines with BR101801 (250 nM) for 16 h, live imaging clearly showed that both cancer cell lines highly accumulated positive signaling of the caspase 3/7 probe (**Figure 3B**, active CAS3/7), with the formation of typical apoptotic bodies (**Figure 3B**, DIC). In addition, flow cytometry analysis showed that BR101801 treatment increased phosphatidylserine exposure to the outer plasma membrane of cancer cells (annexin 5-positive) and membrane rupture (PI-positive) (**Figure 3C**). Fur-

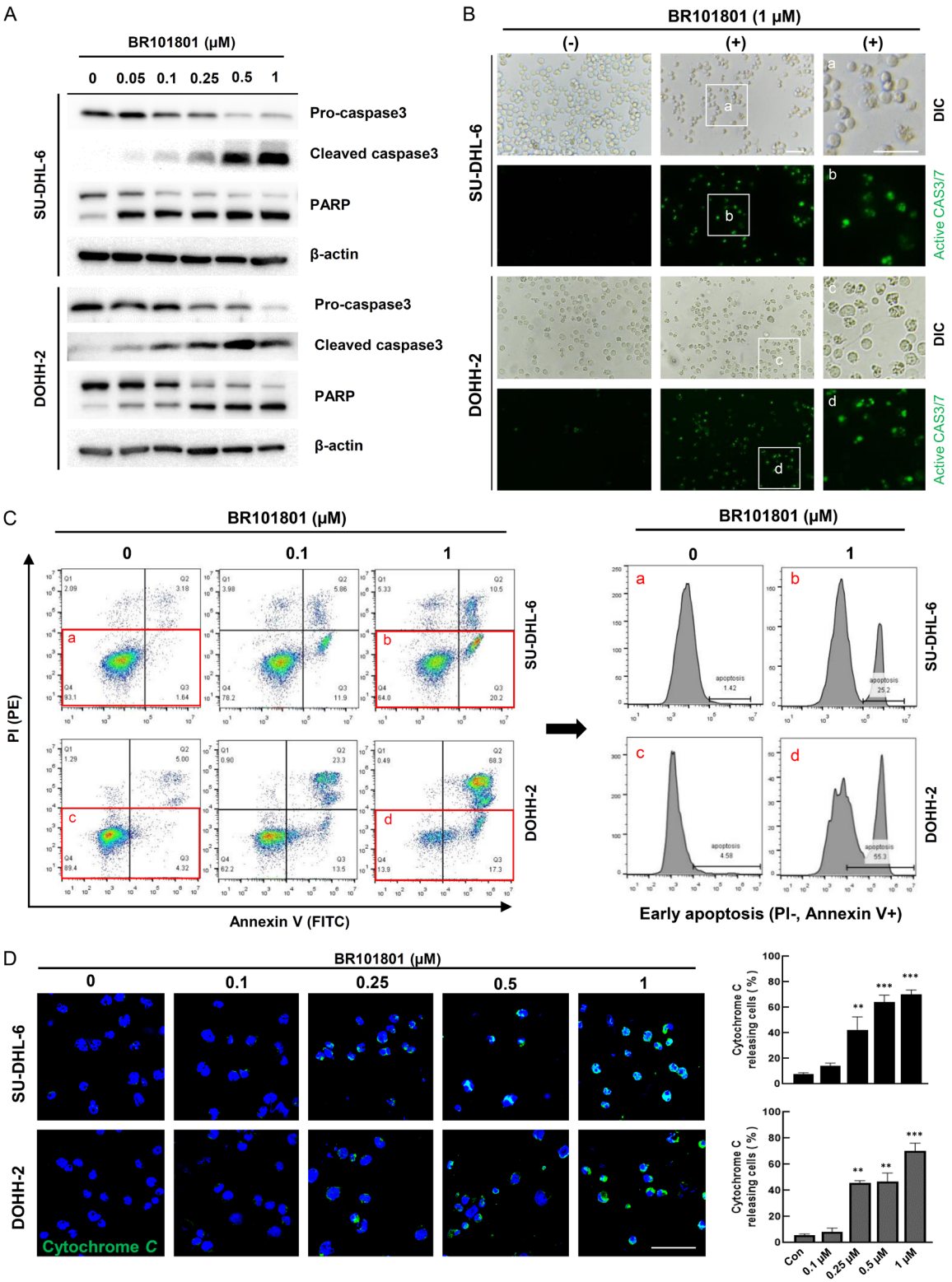

Figure 3. BR101801 has an anti-cancer effect by inducing apoptosis in DLBCL. A. SU-DHL-6 cells were treated with serial concentrations of BR101801 for 24 h, followed by Western blot analysis with the indicated antibodies. B. Active caspase 3/7 probe live imaging. SU-DHL-6 cells and DOHH-2 cells were treated with an active caspase 3/7 probe after treatment with 250 nM BR101801 for 16 h. C. Flow cytometric analysis of Annexin V and PI in SU-DHL-6 cells and DOHH-2 treated with BR101801. D. DLBCL cells were stained with anti-Cytochrome C antibody following treatment with BR101801 for 16 h. Scale bar, 30 μm. Data are presented as the mean ± S.D. (**P < 0.01, ***P < 0.001 vs Con).

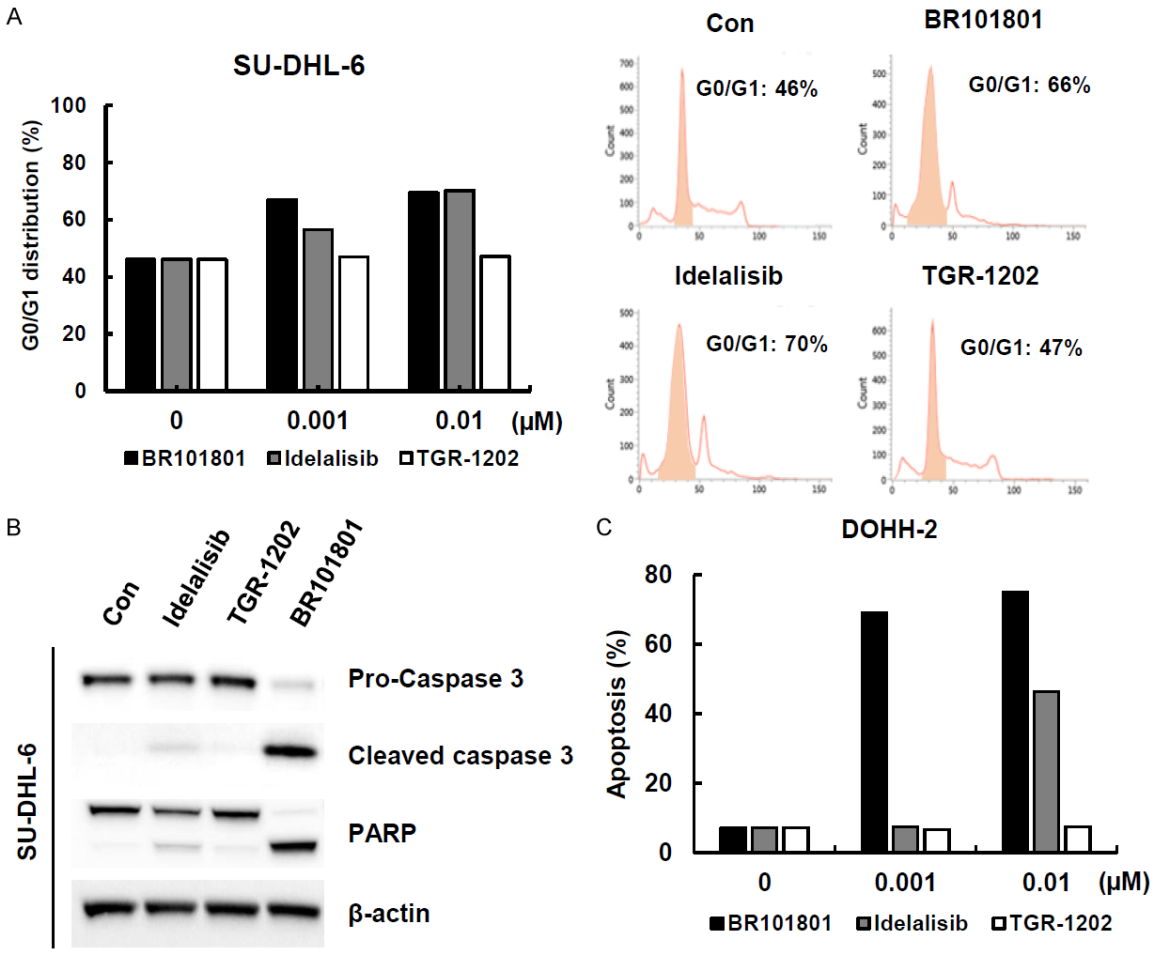

Figure 4. BR101801 shows superior anti-cancer effects than verified PI3K inhibitors idelalisib or TGR-1202. A. BR101801 treatment increased cell population in G0/G1 more than idelalisib or TGR-1202. B. SU-DHL-6 cells were treated with idelalisib, TGR-1202, and BR101801 (1 nM) for 24 h followed by Western blot analysis with the indicated antibodies. C. BR101801 induced more apoptosis in DOHH-2 cells than idelalisib or TGR-1202.

thermore, the release of Cytochrome C significantly increased in both SU-DHL-6 and DOHH-2 cell lines in a dose-dependent manner (**Figure 3D**). These results indicated that BR101801 exerts an anti-cancer effect by inducing caspase 3-dependent apoptosis in DLBCL cells.

BR101801 shows superior anti-cancer effects than verified PI3K inhibitors idelalisib or TGR-1202

To evaluate the anti-cancer effect of BR101801, we compared its effects with those of the PI3K inhibitors idelalisib or TGR-1202. BR101801 treatment increased the cell population of G0/G1 cells more than idelalisib or TGR-1202 (**Figure 4A**). Furthermore, BR101801 also induced apoptosis at a much lower concentration than TGR-1202 or idelalisib (**Figure 4B** and **4C**). These results reveal that BR101801 has superior anti-cancer effects compared to idelalisib or TGR-1202, FDA-approved PI3K inhibitors, strongly supporting the potential of BR101801 as an anti-cancer drug.

BR101801 inhibits tumor growth by decreasing both c-Myc and Mcl-1 levels in DLBCL cells

c-Myc stabilization followed by PI3K activation is essential for DLBCL progression [21, 22], and PI3K suppression has recently been reported to overcome Mcl-1 dependent resistance to Venetoclax in double-hit DLBCL [23]. As expected, BR101801 suppressed c-Myc expression in a dose-dependent manner. Notably, BR101801 also suppressed the expression of Mcl-1, which is a potential target for overcoming Venetoclax resistance (**Figure 5A**). This observation is con-

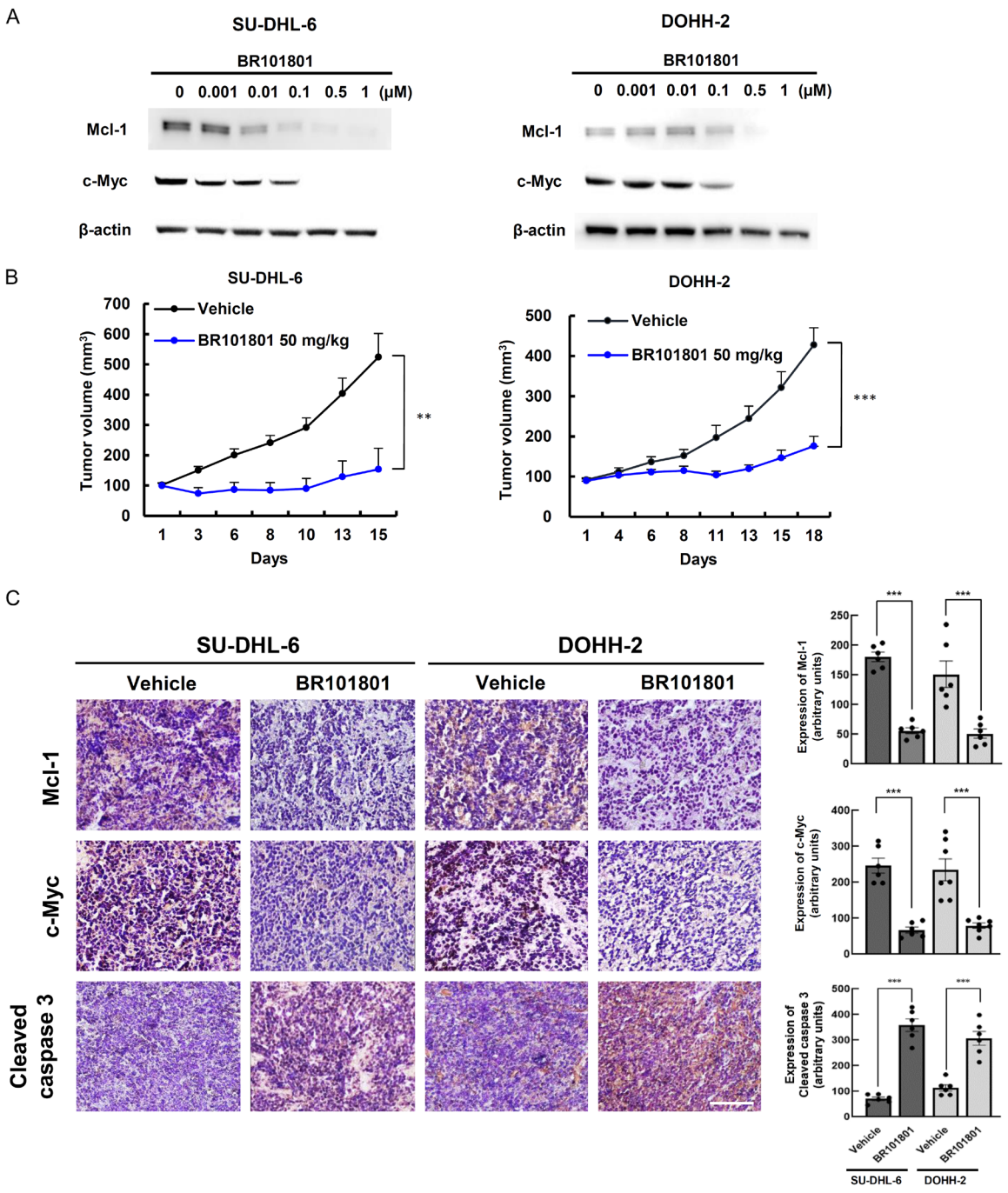

Figure 5. BR101801 inhibits tumor growth by decreasing both Mcl-1 and c-Myc levels in DLBCL cells. A. SU-DHL-6 and DOHH-2 cells were treated with serial concentrations of BR101801 for 24 h, followed by Western blot analysis with the indicated antibodies. B. In vivo validation of BR101801 using SU-DHL-6 and DOHH-2 cells xenograft models. When the tumor size reached 100 mm^3, BR101801 was orally administered daily through oral gavage. C. Histological analysis of xenograft tumor tissue using hematoxylin and eosin (H&E) staining, immunohistochemical detection of Mcl-1, c-Myc, and cleaved caspase 3. Data are presented as the mean ± S.D. ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

sistent with previous reports that Mcl-1 and c-Myc expression is dependent on the PI3K pathway [21, 24]. To validate the antitumor effect of BR101801 in vivo, a xenograft model was established using SU-DHL-6 and DOHH-2 cells. Compared to the control group, the

BR101801 treatment group exhibited significant tumor regression (**Figure 5B**). Similar to the in vitro results, BR101801 treatment inhibited tumor growth and decreased the expression of Mcl-1 and c-Myc in both SU-DHL-6 and DOHH-2 xenograft tumors (**Figure 5C**). These results reveal that BR101801 has a substantial antitumor effect in vivo. Furthermore, as BR101801 can inhibit both Mcl-1 and c-Myc expression in vivo, it is expected to be used in combination with Venetoclax for double-targeting DLBCL to complement Venetoclax resistance.

The combination of BR101801 and Venetoclax has a significant synergistic antitumor effect

When the GI50 of BR101801 was compared with those of ibrutinib and idelalisib for the treatment of DLBCL, BR101801 showed a much lower GI50 than the other drugs (**Figure 6A**). In addition, BR101801 effectively inhibited the expression of Mcl-1 and c-Myc compared to ibrutinib or idelalisib, which was more clearly observed when it was combined with Venetoclax (**Figure 6B**). Moreover, the combination of Venetoclax with BR101801 induced clearer PARP cleavage than the combination of Venetoclax with ibrutinib or idelalisib (**Figure 6B**). These results indicate that BR101801 shows promise as a combination therapy option that could synergize with Venetoclax. To this end, the efficacy of the combination therapy was evaluated using xenograft animal models. Compared with treatment with either agent alone, the combination of BR101801 and Venetoclax significantly reduced tumor growth (**Figure 6C**). Importantly, this combination therapy exhibited an antitumor effect in a late-stage xenograft model (**Figure 6D**), strongly suggesting that c-Myc/Bcl-2/Mcl-1 triple targeting through the combination of BR101801 and Venetoclax could be a great clinical therapeutic option for double hit DLBCL. The combination of BR101801 and ibrutinib showed additive antitumor effects in both early and late xenograft models (Figure S1), suggesting that BR101801 can also be administered with other drugs targeting DLBCL.

Discussion

The PI3K signaling pathway is known to play important roles in cancer cell proliferation and tumorigenesis in DLBCL [25]; therefore, it has attracted attention as a potential therapeutic target for DLBCL treatment [11]. We validated the antitumor effect of BR101801, a novel PI3K inhibitor. Compared to other PI3K inhibitors, such as idelalisib and TGR-1202, BR101801 more effectively inhibited DLBCL proliferation and cell arrest. Furthermore, BR101801 induced apoptosis more than idelalisib or TGR-1202, suggesting that BR10180 could be a good option for establishing a therapeutic strategy for PI3K pathway-activating cancer types such as DLBCL.

The activation of survival signals and/or anti-apoptotic pathways is observed in various cancer types and has significant effects on the abnormal proliferation and malignancy of cancer cells [26]. The double-hit DLBCL population that exhibits rearrangement and overexpression of both c-Myc and Bcl-2 has a malignant phenotype and drug resistance, and is thought to be a major cause of DLBCL recurrence [27]. As PI3K is a major upstream signal regulating c-Myc expression, a combination of a PI3K inhibitor with Venetoclax, the first approved BCL-2 inhibitor, is being considered for double-hit DLBCL targeting [17]. However, a recent clinical study of Venetoclax monotherapy in patients with relapsed/refractory DLBCL showed disappointing response rates [15]. Interestingly, related studies have shown that increased levels of Mcl-1, another anti-apoptotic factor, may be closely associated with resistance to Venetoclax [16]. This evidence indicates that c-Myc/Bcl-2/Mcl-1 triple targeting should be considered a therapeutic strategy for double-hit DLBCL.

In this respect, BR101801 is expected to offer significant advantages when used in a combination therapy targeting double-hit DLBCL. Our study revealed that treatment with BR101801 resulted in downregulation of both Mcl-1 and c-Myc levels more effectively than with other PI3K inhibitors such as idelalisib or TGR-1202, which may explain why BR101801 is superior in inhibiting cell proliferation and inducing apoptosis in DLBCL. Consistent with these in vitro results, BR101801 also suppressed tumor growth, with a reduction in both Mcl-1 and c-Myc levels in DLBCL xenograft tumors. Therefore, BR101801 could also enhance the antitumor effect of Venetoclax synergistically.

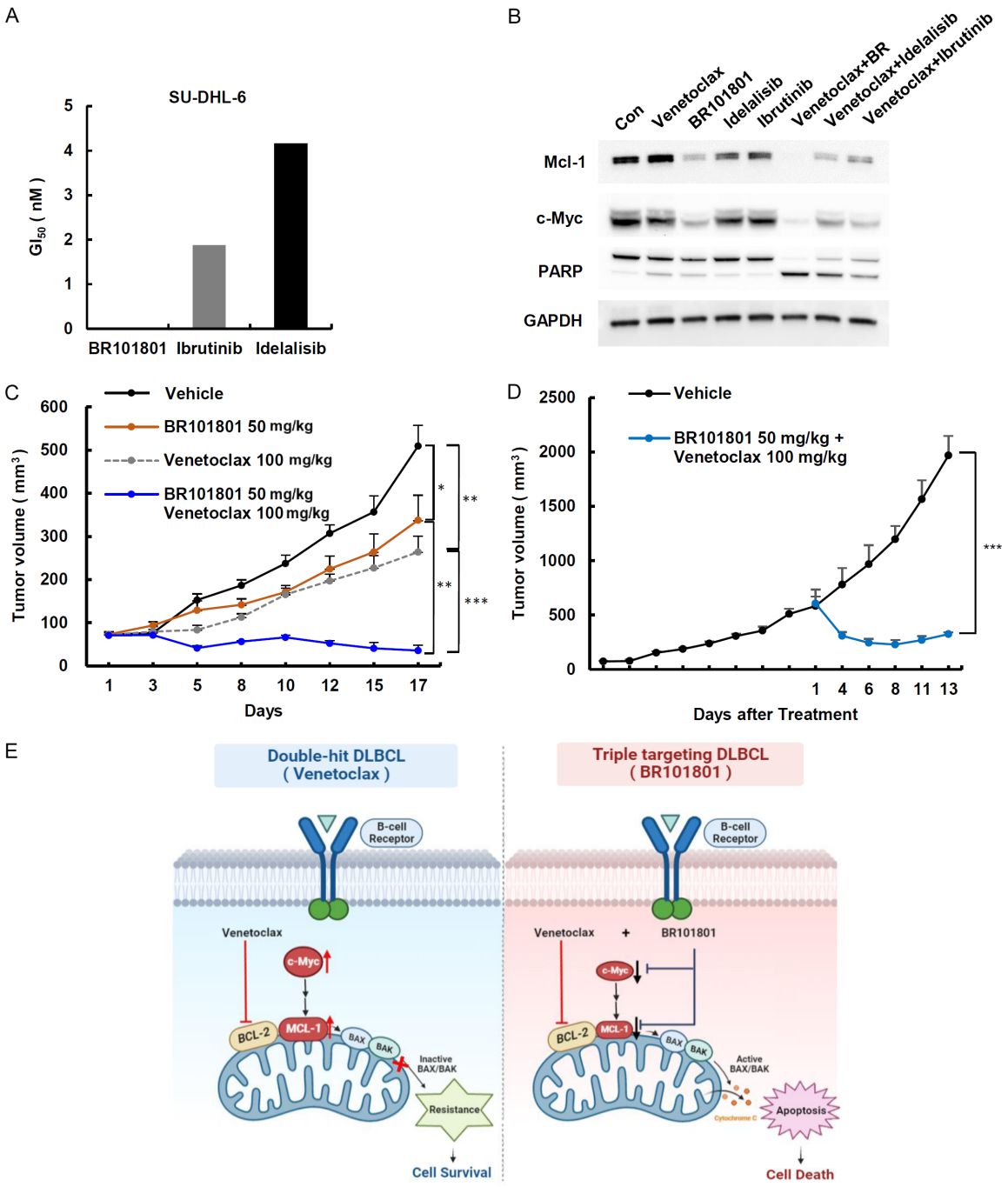

Figure 6. The combination of BR101801 and Venetoclax has a significant synergistic antitumor effect. A. GI50 of BR101801, ibrutinib, and idelalisib for the SU-DHL-6 cell line. In vitro anti-cancer effects of BR101801 were confirmed in various cell lines. B. In vitro synergy effect of the combination of with BR101801 and Venetoclax. SU-DHL-6 cells were treated with BR101801 (100 nM), ibrutinib (100 nM), idelalisib (100 nM), or/and Venetoclax (10 nM) for 24 h. C. Comparison of the effects of BR101801 and Venetoclax as a monotherapy and combination therapy. DOHH-2 xenografts were treated with BR101801 (50 mg/kg, PO, QD) and/or Venetoclax (100 mg/kg, PO, QD). D. The antitumor effect of BR101801 and Venetoclax combination therapy in a late-stage tumor model. DOHH-2 xenografts were treated with a combination of BR101801 (50 mg/kg, PO, QD), with Venetoclax (100 mg/kg, PO, QD). E. Schematic showing the mechanism of action of BR101801 in DLBCL. Data are presented as the mean ± S.D. ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Collectively, BR101801 significantly inhibited DLBCL growth by blocking the PI3K/c-Myc/Mcl-1 pathway, and its combination with Venetoclax could be a very good option for dou-

ble-hit DLBCL treatment through triple-targeting of c-Myc/Bcl-2/Mcl-1.

Acknowledgements

This study was supported by an Inha University Grant.

Disclosure of conflict of interest

None.

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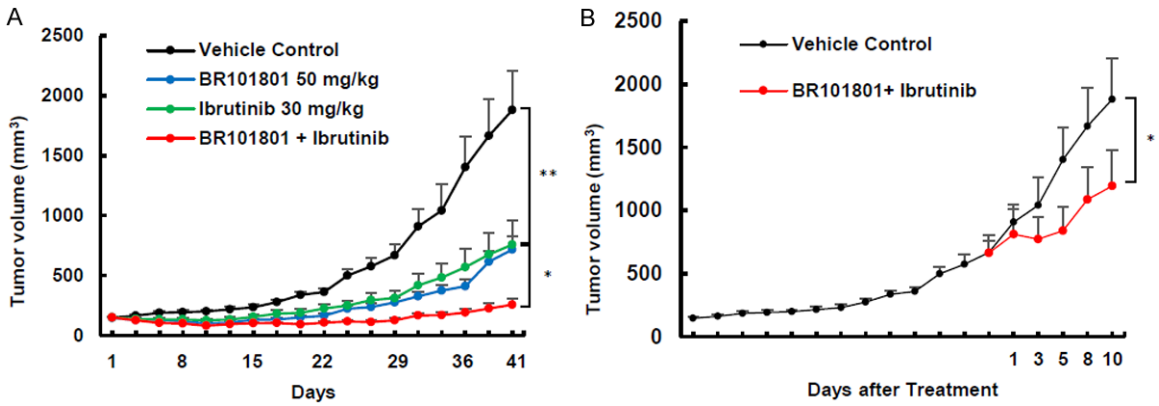

Figure S1. The combination of BR101801 and ibrutinib has an additive antitumor effect. A. Comparison of the effects of BR101801 and ibrutinib as monotherapy and combination therapy. DOHH-2 xenografts were treated with BR101801 (50 mg/kg, PO, QD) and/or ibrutinib (30 mg/kg, PO, QD). B. The antitumor effect of BR101801 and ibrutinib combination therapy in late-stage tumor model. DOHH-2 xenografts were treated with the combination of BR101801 (50 mg/kg, PO, QD) and ibrutinib (30 mg/kg, PO, QD). Data are presented as the mean ± S.D. ($^{*}P < 0.05$ and $^{**}P < 0.01$).