Co-targeting Aurora kinase A and Bcl-2 synergistically inhibits the viability in double-hit lymphoma cells

Ling-Zhe Kong1*, Xiao-Hui Jia1*, Zheng Song1, Li-Hua Qiu1, Lan-Fang Li1, Zheng-Zi Qian1, Shi-Yong Zhou1, Xian-Ming Liu1, Xi-Bao Ren2, Bin Meng3, Kai Fu4, Ping Wang5, Xian-Huo Wang1, Hui-Lai Zhang1

Departments of 1Lymphoma, 2Biotherapy, 3Pathology, The Sino-US Center for Lymphoma and Leukemia Research, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center of Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin's Clinical Research Center for Cancer, Tianjin 300060, China; 4Departments of Pathology and Microbiology, and Internal Medicine, University of Nebraska Medical Center, Omaha, NE, USA; 5Radiation Oncology, The Sino-US Center for Lymphoma and Leukemia Research, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center of Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin's Clinical Research Center for Cancer, Tianjin 300060, China

Contributions: (I) Conception and design: HL Zhang, XH Wang; (II) Administrative support: HL Zhang, XH Wang, P Wang; (III) Provision of study materials: LZ Kong, XH Jia; (IV) Collection and assembly of data: Z Song, LH Qiu, LF Li, XM Liu, LX Feng; (V) Data analysis and interpretation: LZ Kong, XH Jia, B Meng, K Fu, XB Ren; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*These authors contributed equally to this work.

Correspondence to: Hui-Lai Zhang. Department of Lymphoma, The Sino-US Center for Lymphoma and Leukemia Research, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center of Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin’s Clinical Research Center for Cancer, Tianjin 300060, China. Email: zhlwqg@126.com; Xian-Huo Wang. Departments of Lymphoma, The Sino-US Center for Lymphoma and Leukemia Research, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center of Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin’s Clinical Research Center for Cancer, Tianjin 300060, China. Email: tjzlyy_xianhuow@163.com.

Background: Double-hit lymphoma (DHL) is a rare high-grade B-cell lymphoma characterized by MYC and Bcl-2 or Bcl-6 gene translocations. The treatment of DHL remains a substantial clinical challenge due to remarkably undesirable outcomes. Innovative drugs or combination treatment strategies need to be developed to improve the prognosis of DHL patients. The purpose of this study was to investigate the combination treatment of a novel Aurora kinase A (Aurka) inhibitor, named alisertib (MLN8237) and a selective Bcl-2 inhibitor, named ABT-199 in vitro, and to explore the underlying mechanisms in human DHL cell lines.

Methods: Cell proliferation was assessed using MTS assay in DHL cells treated with alisertib and ABT-199. Synergistic effects between two drugs were analyzed based on combination index (CI) values. Cell cycle and apoptosis were detected through flow cytometry. The expression of drug-targeted proteins, cell-cycle proteins as well as apoptosis-related proteins were detected by Western blot.

Results: Alisertib and ABT-199 alone exhibited a relatively modest cytotoxicity in a concentration- and time-dependent manner, but the combination treatment produced stronger antitumor efficacy and synergistically suppressed the DHL cell growth. Otherwise, the combination treatment between alisertib and ABT-199 arrested the cell cycle in the G2/M phase at respectively lower concentrations (2/5 IC50), while the higher concentrations (3/5 IC50) resulted in a striking increase of cell apoptosis. Further studies demonstrated that both alisertib and ABT-199 downregulated the expression of cyclin-dependent kinase 1 (CDK1) and cyclin B1, but upregulated the p21 and p53 expression. The combination treatment enhanced the expression of cell intrinsic apoptotic proteins, including cleaved Poly ADP-ribose polymerase (PARP) and caspase-3. In addition, the combination treatment almost completely inhibited the expression of drug-targeted proteins, including MYC and Aurka phosphorylation. However, alisertib and ABT-199 did not alter the Bcl-2 expression.
Introduction

Double-hit lymphoma (DHL) has been defined as high grade B cell lymphomas with MYC and Bcl-2 or Bcl-6 translocations according to the 2016 World Health Organization classification (1). DHL has been becoming the focus of intense investigation due to its intractable clinical behaviors and dismal prognoses. Most DHL cases possess concomitant translocations of MYC and Bcl-2 (58–87%), but few harbor MYC/Bcl-6 dual rearrangements (2-5). DHL patients tend to have a plurality of unfavorable prognostic parameters, including high tumor burden, elevated serum lactate dehydrogenase levels, advanced stage, high International Prognostic Index score, and extranodal involvement (6). The conventional immunochemotherapy regimen (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) and aggressive therapeutic regimens (R-DA-EPOCH, R-Hyper CVAD and R-CODOX-M/IVAV) did not remarkably improve the patient outcomes, especially overall survival (OS), with median OS of no more than 2 years (7,8). A majority of patients succumb to refractory or relapsed diseases after a short remission.

The main characteristics of DHL are the translocations of MYC and Bcl-2, and their dysfunction has indeed been confirmed to contribute to malignant cell growth and pathogenesis in DHL (6,9,10). One of the alternative strategies is the combination treatment of two promising inhibitors of MYC and Bcl-2 for DHL patients (11,12). MYC directly regulates Aurora kinase A (Aurka), which in turn expedites MYC transcription by binding to its promoter, thus forming a feedback loop between MYC and Aurka (13,14). Aurka is a member of highly conserved serine/threonine Aurora kinase family, and participates in the regulation of multiple cell cycle aspects, particularly in the G2/M phase. Aurka is highly elevated in aggressive lymphomas, such as MYC-driven B-cell lymphomas and other types of lymphoma with high growth fractions, and it is also correlated with disease activity and prognosis (13,15,16). Alisertib (MLN8237), a highly specific second-generation Aurora inhibitor, has been demonstrated to have some encouraging preclinical activities and been underway in clinical trials for the treatment of several tumors (17). Bcl-2 regulates the intrinsic apoptosis pathway and therefore stimulates chemoresistance (18). Strategies that antagonize Bcl-2 function have been widely developed due to the emergence of a number of promising BH3 mimetics. ABT-199 (Venetoclax), a selective third-generation BH3 mimetic, has gained FDA approval as a monotherapy for chronic lymphocytic leukemia (19). Studies have reported the synergistic antitumor effects between ABT-199 and other agents, suggesting that the potential value of the combination therapy between ABT-199 and other novel inhibitors may be observed in some highly invasive tumors (20,21). Moreover, ABT-199 alone has also exerted preclinical efficacy in murine MYC-driven lymphoma models (22).

Herein, the purpose of this study was to evaluate the combination treatment of alisertib and ABT-199 in DHL cells. This was the first time to demonstrate that synergistic effects of Aurka inhibitors and Bcl-2 inhibitors, which was potentially therapeutic strategy for DHL lymphoma.

Methods

Cells and reagents

The DHL cell lines used in this study (DOHH2 and VAL) were provided by Dr. Kai Fu (University of Nebraska Medical Center, NE, USA), which had been validated by STR DNA finger printing (Figures S1,S2). DOHH2 was maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA). VAL was cultured in Iscove’s modified Dulbecco’s...
medium (IMDM, GE Healthcare, Illinois, USA) containing 10% FBS. All media were supplemented with 1% penicillin/streptomycin, and cells were incubated under 37 °C humid condition with 5% CO₂. Alisertib and Nocodazole (Noc) were purchased from Selleck Chemicals (Houston, TX, USA). ABT-199 was supplied by MedChem Express (NJ, USA).

**Cell proliferation assay**

DHL cells were treated with ABT-199 or alisertib at concentrations ranging from 10 to 10,000 nM for 24, 48 and 72 h. Changes in cell viability were evaluated using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA, Cat.#G3580) following the manufacturer's protocol. The 50% inhibitory concentration (IC50) values were calculated utilizing GraphPad Prism 5.0 software.

**Drug combinations and synergy evaluation**

Cells were incubated with single or combination treatment of alisertib and ABT-199 at concentrations of 1/5–5/5 IC50 for 48 h. MTS assay was then performed as described above. The effects of the combination treatment were calculated by the CompuSyn software using the Chou-Talalay combination index (CI) and isobologram methods according to the median-effect principle (23). The combination effect was designated synergism (CI <1.0), addition (CI =1.0), or antagonism (CI >1.0).

**Cell cycle assay**

DHL cells were treated with ABT-199 or/and alisertib at 2/5 IC50 for 48 h, then washed and resuspended in PBS, and fixed by dropwise addition of ice-cold absolute ethanol to a final concentration of 70%. Fixed cells were stored at 4 °C overnight, pelleted and incubated with RNase A (Solarbio, China, final concentration was 0.2 mg/ml), and propidium iodide (PI, Solarbio, China, final concentration was 50 μg/mL) for 30 min at 37 °C protected from light. Cell cycle was analyzed on a FACSCelesta flow cytometer (BD Bioscience, CA, USA) by ModFit LT 4.1 software.

**Apoptosis assay**

Cells were treated with ABT-199 or/and ALS at 3/5 IC50 for 48 h, then were rinsed with cold PBS once. After centrifugation at 300 xg for 5 min, cells were resuspended in 200 μL of binding buffer (BD Bioscience, CA, USA), and then 3 μL of Annexin V-FITC and 3 μL of PI were added. The samples were analyzed by FACSCelesta post incubation for 15 min in the dark at room temperature (RT). Results were analyzed with FlowJo V10 software.

**Western blot analysis**

Treated cells were harvested in RIPA lysis buffer with 1 mM PMSF and phosphatase inhibitor cocktail II. The cell lysates were centrifuged at 12,000 xg at 4 °C for 10 min. The protein concentration was measured using the BCA method (Thermo Fisher Scientific, MA, USA). A 30 μg of total protein was separated by 10% SDS-PAGE gel electrophoresis and subsequently transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany) at 260 mA for 2 h. Then, the blot was blocked in 5% bovine serum albumin for 1 h at RT and incubated with the specific primary antibodies at 4 °C overnight. After washing in tris-buffered saline–Tween thrice for 10 min, the blot was probed with 1:5,000 dilution of goat anti-rabbit IgG or anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, MO, USA) for 1h at RT. Signals were detected using a chemiluminescence reagent Immobilon (Merck Millipore, Darmstadt, Germany). Signal intensity was obtained by Image Studio Lite software. For the detection of Aurka phosphorylation, the cells were first synchronized by 200 ng/mL Noc for 10 h, then were untreated or treated with alisertib or/ and ABT-199 for 1 h. Antibodies used, including rabbit polyclonal anti-MYC (1:1,000, Cat.#10828-1-AP), rabbit polyclonal anti-Bcl2 (1:2,000, Cat.#12789-1-AP), rabbit polyclonal anti-p53(1:1,000, Cat.# 60214-1-Ig), mouse monoclonal anti-p21(1:10,00, Cat.# 60214-1-Ig) and rabbit polyclonal anti-MCL1(1:1000, Cat.#16225-1-AP) were purchased from ProteinTech Group, Inc. (Hubei, China). Rabbit monoclonal anti-phospho-Aurka (Th288, 1:1,000, Cat.#3079), rabbit monoclonal anti-Aurka (1G4, 1:1,000, Cat.#4718), mouse monoclonal anti-Caspase-3 (1:1,000, Cat.#9668), mouse monoclonal anti-cleaved PARP (1:1,000, Cat.#9546), mouse monoclonal anti-CDK1/cdc2 (1:1,000, Cat.#9116), and rabbit monoclonal anti-Cyclin B1(1:1,000, Cat.#12231) were obtained from Cell Signaling Technology (MA, USA). Mouse monoclonal anti-β-actin (1:2,000, clone AC-15, A1978) was supplied by Sigma-Aldrich and used as an endogenous protein for normalization.
Statistical analysis

All assays were performed three times, and data were displayed as the mean ± standard deviation (SD). Differences were analyzed by one-way ANOVA, using IBM SPSS Statistics 20 software. P<0.05 was considered significant.

Results

**ABT-199 and alisertib inhibited the DHL cell proliferation**

Cell proliferation was examined in DHL cells after exposure to ABT-199 or alisertib. Figure 1 showed that ABT-199 and alisertib suppressed the cell viability in a dose- and time-dependent manner in DOHH2 and VAL cells, respectively. The IC50 values of the two drugs ranged from 15 nM to over 1,000 nM for 24, 48 and 72 h (Table 1), suggesting that ABT-199 and alisertib inhibited the cell proliferation in DHL cells.

**ABT-199 combined with alisertib synergistically inhibited the DHL cell proliferation**

DOHH2 and VAL cells were exposed to alisertib combined with ABT-199 at a panel of concentrations (1/5–5/5 IC50). Figure 2A revealed that the combination treatment promoted stronger inhibitory activity and synergistically suppressed the DHL cell growth compared to either agent alone. Median dose-effect analysis of the combination treatment suggested that ABT-199 and alisertib had synergistic cytotoxic effects (CI <1.0) in DHL cells (Figure 2B).
Figure 2 Combination treatment between ABT-199 and alisertib synergistically inhibited cell viability in DHL cells. (A) The inhibition rate of cell proliferation in DOHH2 and VAL cells treated with ABT-199 and/or alisertib for 48 h. Shown are mean ± SD; (B) combination index (CI) and fractional effect (Fa) of ABT-199 and alisertib are plotted by the CompuSyn program for drug interactions. CI values <1.0 indicate synergism.

ABT-199 combined with alisertib induced G2/M phase arrest in DHL cells

Cell cycle analyses were further performed to characterize the antitumor efficacy of the combination treatment in DOHH2 and VAL cells. As shown in Figure S3, the combined treatment tended to induce more cell accumulation in G2/M phase. These results suggested that alisertib combined with ABT-199 seemed to boost G2/M phase arrest in DHL cells.

ABT-199 and alisertib regulated p53/p21 signaling in DOHH2 cells

To study the mechanisms of inducing G2/M phase arrest, we first analyzed the expression of key cell-cycle regulators, including CDK1/cdc2 and cyclin B1, in DOHH2 cells after treatment with ABT-199 and alisertib at 0.1 and 1 μM for 24 h. Figure 3 displayed that the expression of CDK1/cdc2 and cyclin B1 was significantly reduced by alisertib. Strikingly, we also observed that ABT-199 could downregulate the expression of CDK1/cdc2 and cyclin B1. To further investigate the underlying upstream mechanism to regulate the CDK1/cdc2 and cyclin B1, we next detected the expression of p53 and p21. We found that the expression of p53 and p21 were upregulated by both alisertib and ABT-199, suggesting that the two agents regulated p53/p21 signaling in DOHH2 cells.
ABT-199 combined with alisertib synergistically induced intrinsic apoptosis in DHL cells

Apoptosis assays were performed to determine whether cell apoptosis was engaged in the cytotoxicity of alisertib and ABT-199 in DHL cells. As shown in Figure S4A,B, the combination treatment induced a consistently significant increase in the percentages of apoptotic cells compared to single-agent treatment (P<0.05). We then examined whether the cell intrinsic apoptotic pathway was regulated by alisertib and ABT-199. Figure S4C revealed that the combination treatment elevated the expression of cleaved caspase-3 and PARP, which was not evidently observed in DHL cells after single-agent treatment. In addition, due to that MCL1 expression had been reported to be a synergistically apoptotic mechanism during the combination treatment between ABT-199 and other drugs (24), MCL1 expression was also detected after exposure to alisertib. Our results showed that alisertib did not downregulate the MCL1 expression (Figure S5).

Meanwhile, the combination treatment also triggered cell morphologic changes in accordance with an apoptotic phenotype characterized by cell shrinkage, chromatin condensation, and membrane bubbling in DHL cells under light microscopy (data not shown).

ABT-199 combined with alisertib enhanced the inhibition of targeted protein expression

The drug-targeted protein levels were further assessed to understand the mechanisms of alisertib and ABT-199 in DHL cells. Figure 4 showed that there was a remarkable decrease in MYC expression upon combination treatment. Unexpectedly, changes of Bcl-2 expression were not observed in DOHH2 and VAL cells. Some studies had shown that auto-phosphorylation of Aurka was a primary requisite for kinase activity on T288 site (25-27). Therefore we evaluated the alterations of p-Aurka on T288 site in DHL cells. Results showed that the levels of p-Aurka were slightly downregulated in cells treated with ABT-199, and were obviously decreased in cells treated with alisertib. The combination treatment further amplified the effects.

Discussion

So far, the standard of care is lacking for DHL. Intensified chemotherapy including R-DA-EPOCH is the preferred treatment protocol in multiple cancer centers. R-DA-EPOCH improved the progress-free survival (PFS) without OS benefit for DHL patients (28,29). Thus, novel alternative treatment modalities should be investigated to improve the prognosis of these high-risk patients (30). Targeted therapies, such as MYC and Bcl-2 based on the underlying genetic lesions of DHL, have
been comprehensively explored due to their oncogenic cooperation (31). Preclinical data also suggested that targeting single oncogene (MYC or Bcl-2) might be inadequate for this lymphoma subtype (32).

MYC is an essential transcription factor which modulates diverse cellular functions, including cell proliferation, cell cycle, migration, differentiation, and metabolism (33,34). Although pharmacological attempts to directly inhibit oncogenic MYC have not borne fruit (35), the improved appreciation of MYC regulation has led to the continuous development of a range of therapeutic approaches targeting MYC-related tumors (36,37). In current study, alisertib was selected for the combination treatment owing to the intimate crosstalk between MYC and Aurka. Aurora kinase is essential for the tumor maintenance of MYC-associated lymphoma (12), whereas blocking Aurora enzyme activity stimulates apoptosis in a MYC-driven lymphoma murine model (13). Thus, simultaneous inhibition of Aurka and Bcl-2 may be required for optimal anti-lymphoma activity in DHL.

Aurka inhibitors have displayed synergistic effects in combination treatment with vincristine and rituximab for hematological malignancies (38,39), and alisertib has also exhibited preliminary clinical efficacy in relapsed/refractory non–Hodgkin lymphoma (40). In our study, the combination treatment with ABT-199 and alisertib exerted synergistic activity in the inhibition of cell viability, and induction of G2/M phase arrest and cell apoptosis in DHL cells. In particular, the combination treatment induced an enhanced delay in G2/M transition at low concentrations, followed by inducing significant cell apoptosis at higher concentrations. Studies have shown that inactivating Aurka activity led to increased stability of p53 and G2/M arrest in other tumor cells (41,42), which was consistent with our data in DHL cells. However, the role of ABT-199 has been poorly clarified in cell cycle regulation. In this study, we identified for the first time that ABT-199 could boost G2/M phase arrest via modulating the expression of CDK1/cdc2, cyclin B1, p21 and p53.

Taken together, our study showed that co-targeting Aurka and Bcl-2 could represent a novel alternative treatment strategy for DHL. Hence, this combination treatment between alisertib and ABT-199 should be subjected to future clinical trials to overcome specific molecular vulnerabilities in DHL lymphoma.

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Footnote

Conflict of Interest: The authors declared no conflicts of interest.

References


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Figure S1 Validation of DOHH2 cell line identity by STR DNA finger printing. Genomic DNA was extracted from the cell pellets, then was amplified together with positive and negative control. Amplified products were processed using the ABI3730xl Genetic Analyzer. Data were analyzed using GeneMapper4.0 software and then compared with the DSMZ databases for reference matching.
Figure S2 Validation of VAL cell line identity by STR DNA finger printing. Genomic DNA was extracted from the cell pellets, then was amplified together with positive and negative control. Amplified products were processed using the ABI3730xl Genetic Analyzer. Data were analyzed using GeneMapper4.0 software and then compared with the DSMZ databases for reference matching.
Figure S3 Combination treatment between ABT-199 and alisertib induced G2/M phase arrest in DHL cells. (A) A map of cell cycle in DHL cells treated with ABT-199 or/and alisertib for 48 h; (B) frequencies of cells in G1, S and G2/M cell cycle phases are revealed as histograms. Columns, mean of 3 experiments; bars, SD. **, P<0.01, vs. control group.
Figure S4 Combination treatment between ABT-199 and alisertib synergistically regulated intrinsic apoptotic pathway in DHL cells. (A) The percentages of cell apoptosis in DHL cells treated with ABT-199 or/and alisertib for 48 h. The data showed one representative of three independent experiments; (B) early (Annexin V+, PI−) and late (Annexin V+, PI+) apoptosis were calculated from three replicate experiments. Error bars, SD. * and **, P<0.05 and P<0.01, respectively vs. control group; (C) the expression of intrinsic apoptotic proteins in DHL cells treated with ABT-199 or/and alisertib for 48 h. β-actin was used as the internal control.

Figure S5 Immunoblot analysis of the expression of MCL1 in DOHH2 after alisertib exposure. DOHH2 cells were treated with alisertib at 0.1 and 1 μM for 24 h. Protein was extracted and subjected to immunoblotting with antibodies against MCL1 and β-actin, respectively.