Abstract. Background: Bendamustine is effective in B-cell malignancies, including mantle cell lymphoma (MCL), alone and in combination with other agents. This study investigated the combination effect of bendamustine and the Bruton tyrosine kinase (BTK) inhibitor PCI-32765 on MCL cell death and the underlying mechanisms. Materials and Methods: Cytotoxicity was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis was assessed by annexin V/propidium iodide staining and protein expression was analyzed by western blotting. Results: When combined with bendamustine, PCI-32765 showed a synergistic effect on growth inhibition of the MCL cell line Jeko-1. Cleavage of caspase-3 and poly-(ADP-ribose) polymerase was increased, indicating enhanced apoptosis induction. In addition, this combination decreased the protein expression of cyclin D1. Phosphorylated v-akt murine thymoma viral oncogene homolog 1 (AKT) (Ser473) was also downregulated, suggesting a suppression of the phosphatidylinositol 3-kinase/AKT signaling pathway. Conclusion: Combination treatment with bendamustine and a BTK inhibitor may be effective in MCL therapy.

Mantle cell lymphoma (MCL), which accounts for approximately 7% of all non-Hodgkin’s lymphomas (NHL) (1), has a poorer prognosis than other types of NHL. The genetic hallmark of MCL is the chromosomal translocation t(11;14)(q13;q32). This translocation leads to overexpression of cyclin D1, which causes deregulation of the cell cycle at the G1-S phase transition (2). In addition to cyclin D1 dysregulation, aberrant activation of multiple signaling pathways that are involved in cell proliferation, survival, and apoptosis occurs in MCL (3). One such signaling pathway that is aberrantly activated in MCL is the B-cell receptor (BCR) signaling pathway, which is essential for normal B-cell development and pathogenesis of B-cell malignancies. The various components of the BCR pathway are constitutively activated and overexpressed in B-cell lymphomas, including MCL (4-7). We have recently shown that the Bruton tyrosine kinase (BTK) inhibitor PCI-32765 (currently ibrutinib), which is an inhibitor of BCR signaling, has a synergistic effect on growth inhibition of MCL cells and enhances apoptosis induction when combined with the histone deacetylase inhibitor vorinostat (8).

Bendamustine is a multifunctional alkylating agent with a unique structure that contains three elements: a 2-chloroethylamine alkylating group, a benzimidazole ring, and a butyric acid side chain (9). The 2-chloroethylamine alkylating group is common to other alkylating agents, such as cyclophosphamide, chlorambucil, and melphalan; chlorambucil also has a butyric acid side chain. The antitumor activity of bendamustine may be different from other alkylating agents because of its unique benzimidazole ring. However, its precise mechanisms of action have not been fully elucidated. Bendamustine has been investigated both as a single agent and in combination with various agents in patients with NHL including MCL (10-13). In particular, the combination of bendamustine with rituximab demonstrated an improved efficacy and lower toxicity compared to rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) in a randomized trial (14). Treatment strategies containing bendamustine are currently undergoing intensive development.

In the present study, we investigated the efficacy of the combination of bendamustine and the BTK inhibitor PCI-32765 on an MCL cell line.

Materials and Methods

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sulfoxide (DMSO) at a concentration of 10 mM. Two MCL cell lines (Jeko-1, Mino) were obtained from the American Type Culture Collection (Rockville, MD, USA), grown in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 20% (Jeko-1) or 15% (Mino) fetal bovine serum, and maintained in a humid atmosphere with 5% CO₂ at 37°C.

**Analysis of cell proliferation.** Cells were seeded at a density of 1×10³ cells/well in 96-well plates and treated with either vehicle (DMSO) or different doses of bendamustine (5, 10, 25, 50, or 100 μM) for 48 h. For combination drug treatment, cells were simultaneously exposed to bendamustine and PCI-32765 at a fixed molar ratio of the doses that inhibited proliferation by 50% (IC₅₀) of each agent for 24 or 48 h. Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Titer 96 AQUEOUS One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) according to the manufacturer’s instructions. The viability of drug-treated cells was expressed as a percentage of that of the untreated controls. All experiments were carried out in triplicate and repeated three times independently.

**Measurement of apoptotic cells and cell-cycle distribution by flow cytometry.** Jeko-1 cells were treated with bendamustine (12.5 μM) and PCI-32765 (10 μM) alone or in combination for 24 hours. For apoptosis analysis, cells were harvested, washed with phosphate-buffered saline (PBS), and then assessed with annexin V/propidium iodide (PI) staining according to the manufacturer’s instructions (Miltenyi Biotec Inc., Auburn, CA, USA). Annexin V-positive and PI-negative cells were considered the early apoptotic fraction, and cells double-positive for annexin V and PI were considered the late apoptotic fraction. For cell-cycle analysis, cells were harvested, washed with cold PBS, fixed in ice-cold 70% ethanol, and stored at 4°C. Before analysis, the cells were washed again with PBS and suspended in 1 ml of PBS containing 50 μg/ml PI. Following a 30-min incubation on ice, the cell cycle was analyzed. Flow cytometry was performed using an EPICS XL flow cytometer (Beckman Coulter Inc., Brea, CA, USA). All experiments were repeated three independent times.

**Western blotting.** Cells were cultured, treated, and then lysed in RIPA Buffer (Pierce, Rockford, IL, USA) containing both the protease inhibitor Complete and the phosphatase inhibitor PhosSTOP (Roche Diagnostics, Mannheim, Germany). Cell lysates were separated with sodium dodecylsulfate-polyacrylamide gel electrophoresis, and proteins were transferred to polyvinylidene difluoride membranes, that were blocked and probed with primary antibodies. The membranes were then washed with TBS containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The signal was detected using the ECL Prime Western Blotting Detection System and ImageQuant LAS 4000 (GE Healthcare UK, Buckinghamshire, UK). The following primary antibodies were used for western blotting: anti-caspase-3, anti-poly-(ADP-ribose) polymerase (PARP), anti-phospho-ß-akt murine thymoma viral oncogene homolog 1 (AKT) (Ser473), anti-total AKT (Cell Signaling Technology, Beverly, MA, USA), anti-cyclin D1 (Santa Cruz Biotechnology), and anti-ß-actin (Sigma) as a loading control.

**Statistical analysis.** Data are expressed as the means±SD from at least three independent experiments. Excel-Toukei 2010 (Social Survey Research Information, Tokyo, Japan) was used for statistical analysis. A value of p<0.05 was considered statistically significant.

Drug interactions were analyzed based on the median effect method as described by Chou and Talalay (15). CalcuSyn software (Biosoft, Cambridge, UK) was used to calculate the combination index (CI), which is a quantitative measure of the degree of drug interactions. A CI of less than 1.0 indicates synergism, while a value greater than 1.0 indicates antagonism. Data obtained from the cell proliferation assay were used to perform this analysis.

**Results**

**Combination effect of bendamustine with the BTK inhibitor PCI-32765 on cell proliferation in vitro.** Treatment with bendamustine alone inhibited the proliferation of MCL cell lines in a dose-dependent manner (Figure 1A). The IC₅₀ of bendamustine at 48 h was approximately 25 μM for Jeko-1 cells and 16 μM for Mino cells. In our previous study, we examined the cytotoxic effect of the BTK inhibitor PCI-32765 using the same MCL cell lines and determined that the IC₅₀ dose was approximately 20 μM in Jeko-1 cells and 3 μM in Mino cells (8). From these data, we determined a fixed ratio of bendamustine:PCI-32765 for the combination treatment as 1:0.8 in Jeko-1 cells and 5:1 in Mino cells. For both cell lines, combination treatment with bendamustine and PCI-32765 significantly reduced cell viability compared to treatment with bendamustine alone (Figure 1B). These results suggested that PCI-32765 enhanced the cytotoxicity of bendamustine. The CI was determined to assess synergy between bendamustine and PCI-32765. In Jeko-1 cells, this combination treatment yielded CI values less than 1.0, indicating a synergistic effect on inhibition of cell growth (Figure 2A). In contrast, all CI values were higher than 1.0 in Mino cells (Figure 2B), indicating an antagonistic interaction.

**Combination treatment with bendamustine and PCI-32765 induced caspase-dependent apoptosis.** To investigate the mechanisms involved in growth inhibition by combination treatment with bendamustine and PCI-32765 observed in Jeko-1 cells, we performed flow cytometric analysis using annexin V/PI staining. The percentages of all apoptotic cells were determined by the sum of cells in early and late apoptosis. Combination treatment with bendamustine and PCI-32765 significantly increased the apoptotic fraction compared to both treatments alone (Figure 3A). To determine whether the cytotoxicity induced by the combination of bendamustine and PCI-32765 was due to an increase in caspase-dependent apoptosis, cleavage of caspase-3 and PARP was assessed by western blotting. Combination treatment with bendamustine and PCI-32765 markedly increased the cleavage of caspase-3 and PARP compared to both agents alone (Figure 3B). These results suggest that this combination treatment enhances apoptosis induction.
Combination treatment with bendamustine and PCI-32765 reduced the expression of cyclin D1 and phosphorylation of AKT. Many investigations have suggested that cyclin D1, which is overexpressed in MCL, is critical for the pathogenesis of MCL. We examined the expression level of cyclin D1 and found that combination treatment with bendamustine and PCI-32765 reduced cyclin D1 in Jeko-1 cells (Figure 4). Previous studies demonstrated that the phosphatidylinositol 3-kinase (PI3K)/AKT pathway is constitutively activated in some MCL cell lines, as well as in primary MCL cells (16, 17). The PI3K/AKT pathway is a signal transduction pathway that is involved in regulation of the cyclin D1 promoter (18). AKT was phosphorylated on Ser473 in Jeko-1 cells, and we found that AKT phosphorylation (Ser473) was significantly suppressed by combination treatment with bendamustine and PCI-32765 (Figure 4), suggesting inhibition of the PI3K/AKT signaling pathway.

Discussion

MCL is a B-cell malignancy with a poor response to current therapies. Thus, novel therapeutic strategies are needed. Bendamustine and ibrutinib (PCI-32765) are both promising agents for MCL (14, 19, 20), and combination therapies with these drugs are now being tested in clinical trials (NCT01479842, NCT01776840).

In this study, we assessed the combination effects of bendamustine and PCI-32765 on cell proliferation using MCL cell lines. This combination treatment demonstrated a synergistic inhibition of cell growth in Jeko-1 cells due to enhanced apoptosis induction. In contrast, Mino cells were more sensitive to bendamustine alone, but the effect of combination treatment was lower and not synergistic, compared to Jeko-1 cells. Our data suggest that combination treatment with bendamustine and PCI-32765 would be effective against MCL cells, but its efficacy may be cell line-dependent.

We explored the molecular mechanisms of the synergistic effect of this combination and found that the expression of cyclin D1 was markedly reduced by combination treatment (Figure 4). The cyclin D1 promoter, which contains multiple transcription factor-binding sites, is mainly regulated by three signaling pathways: mitogen-activated protein kinase, PI3K/AKT, and Wingless-type MMTV integration site family (WNT) pathways (18). Among them, we focused on the

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Figure 1. Effect of treatment with bendamustine (BDM) alone and in combination with PCI-32765 (PCI) on cell proliferation of mantle cell lymphoma cell lines. A: Cells were treated with different doses of bendamustine alone for 48 hours. Doses that inhibited proliferation by 50% (IC_{50}) were determined with the median effect method using CalcuSyn software. B: Cells were treated with bendamustine (12.5 μM for Jeko-1, 16 μM for Mino) alone or combined with PCI-32765 (10 μM for Jeko-1, 3 μM for Mino) for 24 or 48 hours. Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Results are the mean±SD of three independent experiments performed in triplicate. *p<0.05; **p<0.01 vs. bendamustine alone. DMSO: Dimethyl sulfoxide.
Figure 2. Combination treatment with bendamustine and PCI-32765 induced synergistic inhibition of proliferation of Jeko-1 cells but not of Mino cells. Cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 48-hour treatment with bendamustine alone or combined with PCI-32765 in Jeko-1 cells (A) and Mino cells (B). The combination index (CI) was calculated using CalcuSyn software. A reference line was set at CI=1.0. A CI of less than 1.0 represents a synergistic effect, while a value greater than 1.0 indicates an antagonistic effect.

Figure 3. Combination treatment with bendamustine (BDM) and PCI-32765 (PCI) increased the induction of caspase-dependent apoptosis. Jeko-1 cells were treated with bendamustine (12.5 μM) and PCI-32765 (10 μM) alone or in combination for 24 or 48 h. A: The percentages of apoptotic and dead cells, that were assessed using annexin V/propidium iodide staining, are displayed as the mean±SD of three independent experiments. B: The expression of apoptosis-related proteins after treatment for 48 h was analyzed by western blotting. Arrowheads indicate the cleaved form of caspase-3 and poly-(ADP-ribose) polymerase (PARP), which are hallmarks of apoptosis. β-Actin is shown as a loading control. DMSO: Dimethyl sulfoxide.
Our study demonstrated that the combination of bendamustine and PCI-32765 led to synergistic growth inhibition of Jeko-1 cells that was mediated by suppression of the PI3K/AKT pathway and down-regulation of cyclin D1. Further investigation is required to reveal the precise mechanism involved in this synergy. However, our pre-clinical data could provide a rationale for combination therapy containing bendamustine and ibrutinib for the treatment of MCL.

Figure 4. Combination treatment with bendamustine (BDM) and PCI-32765 (PCI) reduced cyclin D1 expression and phosphorylation of v-akt murine thymoma viral oncogene homolog 1 (pAKT). Jeko-1 cells were treated with bendamustine (12.5 μM) and PCI-32765 (10 μM) alone or in combination for 48 h and then protein expression was analyzed with western blotting. Total AKT and β-actin are shown as loading controls. DMSO: Dimethyl sulfoxide.

involvement of the PI3K/AKT pathway, which plays a critical role in cell proliferation and survival. Previous studies reported that several genes related to the PI3K/AKT pathway are overexpressed and that this pathway is constitutively activated in MCL (16, 17), indicating that the PI3K/AKT pathway may also contribute to the pathogenesis of MCL. As shown in Figure 4, phosphorylated AKT (Ser473), which was highly expressed in the MCL cell line Jeko-1, was reduced by treatment with PCI-32765 alone and was almost totally suppressed by combination treatment with bendamustine and PCI-32765. These results indicate that cyclin D1 expression may be suppressed through down-regulation of the PI3K/AKT signaling pathway, and bendamustine may potentiate the activity of PCI-32765 to down-regulate AKT phosphorylation. These observations are, however, based on one MCL cell line, and the results should be interpreted with caution.

This is the first study that examined the synergistic interaction between bendamustine and PCI-32765 in MCL. Our study demonstrated that the combination of bendamustine and PCI-32765 led to synergistic growth inhibition of Jeko-1 cells that was mediated by suppression of the PI3K/AKT pathway and down-regulation of cyclin D1. Further investigation is required to reveal the precise mechanism involved in this synergy. However, our pre-clinical data could provide a rationale for combination therapy containing bendamustine and ibrutinib for the treatment of MCL.

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