

## Analyses on Activation of NF- $\kappa$ B and Effect of Bortezomib in Canine Neoplastic Lymphoid Cell Lines

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**ABSTRACT.** Lymphoid malignancies, such as leukemia, and many types of lymphoma are common and severe disorders in dogs. Since shortening remission duration caused by resistance to chemotherapy often becomes clinically critical problems, development of novel and effective therapy should be required. The present study investigated the status of NF- $\kappa$ B and effect of its inhibitor, bortezomib, in six canine neoplastic lymphoid cell lines. NF- $\kappa$ B p65 and p50 were detected in the nuclear fraction of GL-1, CLBL-1 and CL-1, suggesting that NF- $\kappa$ B was constitutively activated in the cells. NF- $\kappa$ B p65 was detected in the cytoplasmic fraction of UL-1 and Ema. After incubation with bortezomib, NF- $\kappa$ B p50 and p65 became undetectable in the nuclear fraction of GL-1, CLBL-1 and CL-1, and CLBL-1, respectively, and p65 was clearly degraded in the cytoplasmic fraction of CLBL-1 and CL-1. Bortezomib inhibited the proliferation of all cell lines except Nody-1 in a concentration-dependent manner. The results indicated that constitutive activation of NF- $\kappa$ B could contribute to the proliferation of canine neoplastic lymphoid cells, and bortezomib would have suppressive effects on the NF- $\kappa$ B activation and the proliferation of neoplastic lymphoid cells in dogs.

**KEY WORDS:** canine, leukemia, lymphoma, Nuclear Factor kappa B, proteasome inhibitor.

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Lymphoid malignancies, such as lymphoma and leukemia, are common and severe disorders of canine hematopoietic neoplasms. There are several subtypes of the malignancies, and chemotherapy can be the first-line treatment for them. However, shortening remission duration caused by developing resistance to chemotherapeutic drugs often becomes serious problems during the treatment in canine cases. Therefore, development of novel and effective therapeutic approaches for such cases should be required.

The Nuclear Factor kappa B (NF- $\kappa$ B) is a critical transcription factor to regulate expression of several genes associated with the cell cycle and protection against apoptosis [19]. The NF- $\kappa$ B family proteins consist of 5 subunits, p65/RelA, RelB, c-Rel, NF- $\kappa$ B1 (p50 and its precursor p105) and NF- $\kappa$ B2 (p52 and its precursor p102). They are able to form various hetero- and homo-dimers. It has been known that there are two NF- $\kappa$ B pathways, canonical and alternative pathways that consist of p65-p50 dimers and p52-RelB dimers, respectively. Under normal conditions, NF- $\kappa$ B is inactive in the cytoplasm by binding to its inhibitor, I $\kappa$ B family proteins. Various types of stimuli, such as tumor necrosis factor  $\alpha$  and lipopolysaccharide, can activate NF- $\kappa$ B via phosphorylation of I $\kappa$ B, triggering their ubiquitination and degradation by proteasome 26S. Once I $\kappa$ B is degraded, NF- $\kappa$ B is released and translocated to the nucleus and binds

to specific DNA sequences of its target genes.

NF- $\kappa$ B is considered to be associated with human oncogenesis to activate anti-apoptotic factors such as Bcl-2 families [5, 16, 19]. In several studies on human lymphoma [2, 10, 16, 19], classical pathway or alternative pathway has been activated constitutively and associated with lymphomagenesis. Furthermore, NF- $\kappa$ B inhibitors have been developed and applied to the treatment for some hematopoietic malignancies [3, 5, 8, 13, 18].

Bortezomib is a proteasome inhibitor and utilized for treatment of human multiple myeloma and other hematological malignancies including lymphoma [1, 3, 5, 8, 13]. It has been shown that bortezomib inhibits the proliferation of neoplastic cells by inducing apoptosis and overcomes resistance to chemotherapeutic drugs by inhibiting NF- $\kappa$ B activation. However, the status of NF- $\kappa$ B and effect of bortezomib have been poorly understood in canine lymphoid malignancies.

The purpose of the present study was to investigate the NF- $\kappa$ B status and effect of bortezomib in canine neoplastic lymphoid cells.

### MATERIALS AND METHODS

**Cell culture:** Canine neoplastic lymphoid cell lines of GL-1 [15], UL-1 [20], CLBL-1 [17], CL-1 [14], Nody-1 [9] and Ema [9] were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with penicillin-streptomycin and 10% fetal bovine serum.

**Immunoblot analysis:** Extraction of nuclear and cytoplasmic proteins from lymphoid cells was based upon the method previously described [4]. Nuclear and cytoplasmic extracts of all cell lines that were untreated and treated with bortezomib (10 ng/ml) (LC Laboratories, Woburn, MA, U.S.A.)

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for 24 hr were prepared. Both the extracts (5  $\mu$ g each) of the cells were loaded on 12.5% polyacrylamide gels, transferred onto the PVDF membranes and blocked with 1% skimmed milk at room temperature for 1 hr. The membranes were then incubated with primary antibodies for p65, p50 (both from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A., p65 sc-372, p50 sc-114) [6],  $\beta$ -actin (Cell signaling, Beverly, MA, U.S.A.) and histone H3 (Upstate biotechnology, Santa Cruz, CA, U.S.A.) at 4°C overnight. Histone and  $\beta$ -actin were used as endogenous controls of nuclear and cytoplasmic extracts, respectively. After washing for 2 hr, the membranes were incubated with HRP-labeled secondary antibodies at room temperature for 1 hr. The blots were washed again and visualized with Luminata Forte Western HRP Substrate (Millipore, Temecula, CA, U.S.A.). The series of experiments was performed at least twice to verify the reproducibility. The visualized bands were analyzed semi-quantitatively using the image analysis software ImageJ. Density of each band was measured, and the expression ratio of each NF- $\kappa$ B subunit to  $\beta$ -actin or histone was calculated.

**Cell viability assay:** Each cell line ( $1 \times 10^6$  cells) was treated with various concentrations (0 to 2 ng/ml) of bortezomib for 72 hr. Cells were stained by trypan blue, and viable cells were counted at least twice and viability was calculated based on the reading of parental untreated cells.

## RESULTS

**The analysis of NF- $\kappa$ B activation in canine lymphoid tumor cell lines:** NF- $\kappa$ B p65 (approximately 65 kDa) and p50 (approximately 50 kDa) were detected in the nuclear fraction of GL-1, CLBL-1 and CL-1 cell lines (Fig. 1), suggesting that NF- $\kappa$ B was spontaneously activated in the cell lines. NF- $\kappa$ B p65 was clearly detected in the cytoplasmic fraction of all cell lines except Nody-1 cell line, whereas NF- $\kappa$ B p50 was undetectable in the cytoplasmic fraction of all cell lines. In Nody-1, both NF- $\kappa$ B p65 and p50 were undetectable in both the cytoplasmic and nuclear fractions.

**Bortezomib inhibited constitutive activation of the canonical NF- $\kappa$ B pathway in canine lymphoid tumor cell lines:** All cell lines were exposed to bortezomib (10 ng/ml), and the NF- $\kappa$ B status was analyzed (Fig. 1). In GL-1, CLBL-1 and CL-1 cell lines, NF- $\kappa$ B p50 became undetectable in the nuclear fraction. NF- $\kappa$ B p65 became undetectable in the nuclear fraction of CLBL-1 cell line, and clearly degraded in the cytoplasmic fraction of both CLBL-1 and CL-1 cell lines. The results suggested that bortezomib inhibited NF- $\kappa$ B activation in the cell lines. In UL-1, Nody-1 and Ema cell lines, the NF- $\kappa$ B status seemed to be unchanged. The expression ratio of each detected NF- $\kappa$ B subunit to each parental control decreased after bortezomib treatment (Fig. 3).

**Bortezomib inhibited the proliferation of canine lymphoid tumor cells:** All cell lines were cultured with various concentration (0 to 2 ng/ml) of bortezomib for 72 hr. Bortezomib inhibited the proliferation of all cell lines in a concentration-dependent manner (Fig. 2). Especially, GL-1, UL-1, CLBL-1, CL-1 and Ema cell lines in which NF- $\kappa$ B was activated or expressed (Fig. 1) were more sensitive to bortezomib than

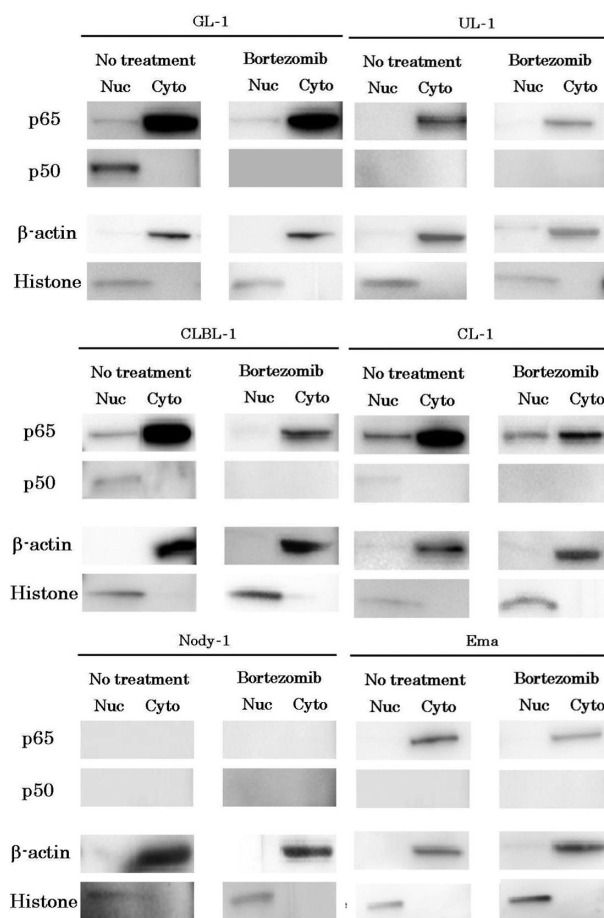


Fig. 1. Detection of constitutive cellular localization of NF- $\kappa$ B subunits, and inhibitory effect of bortezomib (10 ng/ml) on spontaneous NF- $\kappa$ B activation in canine neoplastic lymphoid cell lines. Histone and  $\beta$ -actin were used for endogenous controls of nuclear and cytoplasmic extracts, respectively.

Nody-1 cell line in which NF- $\kappa$ B was undetectable (Fig. 1). From the results,  $IC_{50}$  of bortezomib in all cell lines was calculated (Table 1).

## DISCUSSION

The present study demonstrated that constitutive activation of NF- $\kappa$ B was observed in some canine neoplastic lymphoid cell lines. Several studies on pathogenic roles of NF- $\kappa$ B have been reported in human lymphoid neoplasms [2, 16, 18], whereas few studies have been reported in canine counterparts. The sole previous study in canine lymphoid neoplasms described a part of similar results to the present study that the NF- $\kappa$ B subunit of p65 was detected in the nuclear fraction of GL-1 and CL-1 cell lines [12]. Since the present study revealed that NF- $\kappa$ B subunits of p65 and p50 were apparently expressed in the nuclear fraction of canine neoplastic lymphoid cell lines (GL-1, CLBL-1 and CL-1), NF- $\kappa$ B could be spontaneously activated in canine

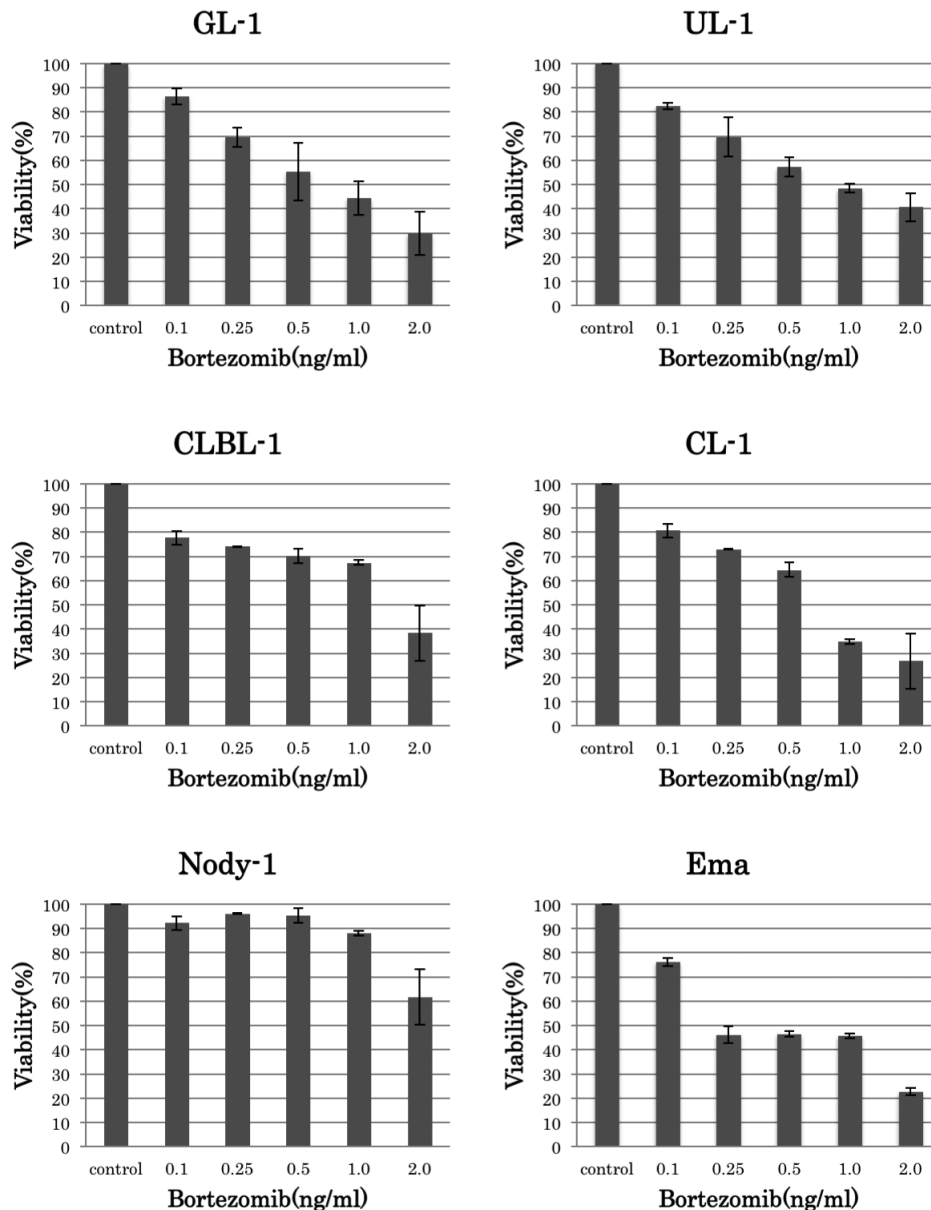


Fig. 2. Inhibitory effect of bortezomib on the proliferation of canine neoplastic lymphoid cell lines. Cells were incubated with serial concentrations of bortezomib. Vertical axis indicates ratios of survival cells toward untreated cells with bortezomib. Horizontal axis indicates serial concentrations of bortezomib.

cases with lymphoid neoplasms. Although expression of both p65 and p50 was detected in GL-1, CLBL-1 and CL-1, p50 was detected in only nucleus and p65 in cytoplasm was more clearly detected than that in nucleus (Fig.1). From the results, p65 was supposed to be highly expressed in the cell lines, and almost all of p50 might bind to p65. Activated p65-p50 dimers could be translocated to nucleus, and the remaining p65 could be left in cytoplasm. The NF- $\kappa$ B pathway has been known to affect many genes involved in the regulation of apoptosis, cell proliferation and cell cycle, and it is considered as a critical molecular target of antitumor

therapy for human lymphoid neoplasms [2, 16, 19]. In the same way, NF- $\kappa$ B would be a therapeutic target of canine lymphoid neoplasms.

Bortezomib is a proteasome inhibitor that can suppress the proliferation of neoplastic cells by downregulating NF- $\kappa$ B activation [18] with hindering degradation of I $\kappa$ B [1]. It has been clinically applied to lymphoid malignancies including multiple myeloma and a part of lymphoma in human patients [3, 8, 13, 16]. The present results show that bortezomib decreased NF- $\kappa$ B subunits in nucleus of some cell lines (Figs. 1 and 3). From a point of view of the pharmacological effect,

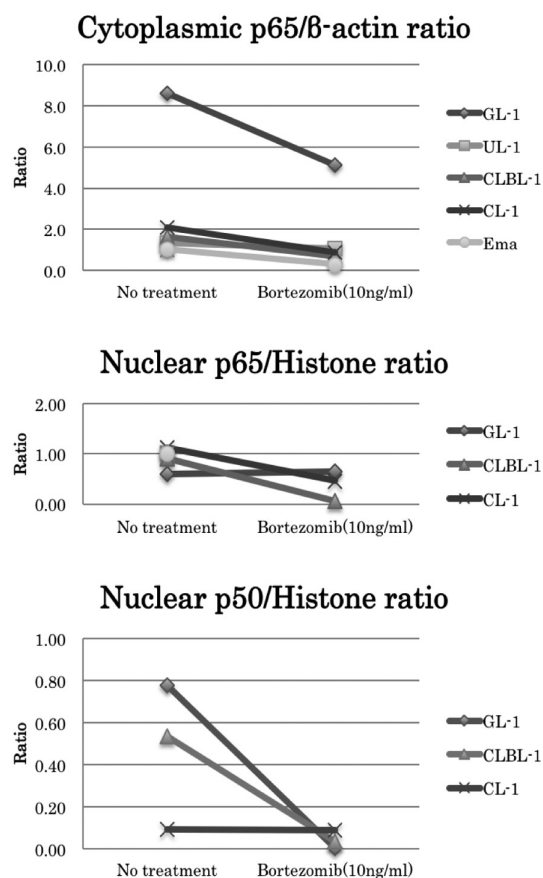


Fig. 3. Effect of bortezomib on the expression of NF- $\kappa$ B subunits. The expression ratios of cytoplasmic p65 to  $\beta$ -actin and nuclear p65 and p50 to histone were analyzed from the immunoblot results by densitometry.

the results suggested that bortezomib impeded translocation of NF- $\kappa$ B from cytoplasm into the nucleus, or expression of NF- $\kappa$ B subunits in the canine neoplastic lymphoid cell lines except Nody-1. Since Nody-1 was the only cell line established from tumor cells in the ascites of a dog with alimentary lymphoma [9], the characteristics might influence the unique NF- $\kappa$ B activity.

And furthermore, bortezomib suppressed the proliferation of the cell lines in a concentration dependent manner (Fig. 2). Although UL-1 and Ema showed relatively lower NF- $\kappa$ B expression than GL-1, CLBL-1 and CL-1 (Fig. 1), the viability of both UL-1 and Ema treated with bortezomib was decreased in a concentration dependent manner (Fig. 2). Considering the results, the alternative pathway might be activated in UL-1 and Ema. Actually, some of human lymphoid malignancies showed activation of only the alternative pathway [10]. The proliferation of Nody-1 cells was scarcely suppressed by bortezomib (Fig. 2), since spontaneous expression of NF- $\kappa$ B subunits was undetectable (Fig. 1). The results suggest that bortezomib can suppress the proliferation of neoplastic lymphoid cells in certain canine cases and downregulate NF- $\kappa$ B activation. Previous studies

Table 1. Estimated IC<sub>50</sub> of bortezomib in canine neoplastic lymphoid cell lines

	IC <sub>50</sub> (ng/ml)
GL-1	0.74
UL-1	0.89
CLBL-1	1.52
CL-1	0.70
Nody-1	2.14
Ema	0.48

of bortezomib on human lymphoid malignancies revealed similar effect in the cell lines [1, 5, 8, 13, 16], and applied effective treatment for clinical patients with resistance to conventional chemotherapy [3, 8, 16].

It has been reported that IC<sub>50</sub> of bortezomib is 0.047  $\mu$ M (equivalent to 18.1 ng/ml) in normal canine peripheral blood mononuclear cells [7]. Since it has been much higher than those in canine neoplastic lymphoid cell lines resulted in the present study (Table 1), neoplastic lymphoid cells especially with constitutive NF- $\kappa$ B activation can be more sensitive to bortezomib than normal blood cells. Thus, bortezomib may be applicable to treatment for canine clinical cases with lymphoid malignancies. A previous study showed that 0.2 mg/kg was the maximal tolerated dosage of bortezomib for dogs [21]. And, another study mentioned that 10 ng/ml of bortezomib supplemented to culturing cell lines was nearly corresponding to the clinical dose used in humans [11]. In the present study, IC<sub>50</sub> of each cell line for bortezomib was lower than 10 ng/ml. Thus, the adequate dosage of bortezomib for dogs is supposed to be lower than that for humans.

The present results indicated that constitutive activation of NF- $\kappa$ B could contribute to the proliferation of canine neoplastic lymphoid cells, and bortezomib would have suppressive effects on the NF- $\kappa$ B activation and the proliferation of neoplastic lymphoid cells in dogs. Since NF- $\kappa$ B activation has been analyzed on only cell lines in the present study, it is necessary to analyze on clinical canine cases with lymphoid malignancies in the further study. Furthermore, it would be valuable to study about the clinical effect of bortezomib for application to the treatment in dogs with lymphoid malignancies.

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