Evaluation of Exportin 1 expression in canine lymphoma cell lines and the antiproliferative effects of the XPO1 inhibitor KPT-335 as a single agent and in combination with doxorubicin or vincristine

犬リンパ腫細胞株を用いたExportin 1の発現とXPO1阻害剤KPT-335の単独およびドキソルビシン・ビンクリスチンとの併用による細胞増殖抑制効果の検討

Joint Graduate School of Veterinary Medicine

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DECLARATION

I hereby declare that the work reported in this research project report has been carried out by the undersigned. I also declare that where reference has been made to the results of other workers, appropriate acknowledgment of the source of information has been made.

March, 2026

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GENERAL INTRODUCTION

Background and Significance

Lymphoma is one of the most common hematopoietic malignancies in dogs, accounting for a substantial proportion of neoplastic diseases encountered in veterinary practice [Vail *et al.*, 2013; Valli *et al.*, 2012]. It is characterized by the uncontrolled proliferation of lymphoid cells, most frequently presenting as multicentric lymphoma involving peripheral lymph nodes, spleen, liver, and bone marrow, and leading to systemic clinical manifestations [Ponce *et al.*, 2010; Valli *et al.*, 2012]. Clinically, affected dogs often exhibit generalized lymphadenopathy, anorexia, lethargy, and organ dysfunction [Zandvliet, 2016; Rocha *et al.*, 2025]. Importantly, canine lymphoma shares remarkable similarities with human non-Hodgkin lymphoma in morphology, immunophenotype, molecular characteristics, and therapeutic response [Avery, 2020; Sapierzyński *et al.*, 2016; Valli *et al.*, 2012]. These parallels have established canine lymphoma as a valuable comparative and translational model, bridging veterinary and human oncology [Oh and Chou, 2023].

Despite advances in veterinary oncology, the prognosis for canine lymphoma remains guarded. Conventional multi-agent chemotherapy protocols, such as CHOP-based regimens (cyclophosphamide, doxorubicin, vincristine, and prednisone), are considered the standard of treatment and often achieve high initial response rates [Kos *et al.*, 2021; Vail *et al.*, 2013]. However, relapse is inevitable in the majority of cases, and the development of chemoresistance limits long-term remission and survival [Zandvliet, 2016]. Median survival times typically range from 6 to 12 months, and only a small subset of patients achieve durable remission [Vail *et al.*, 2013]. These limitations underscore the urgent need for novel therapeutic strategies aimed at enhancing treatment efficacy, overcoming resistance mechanisms, prolonging remission, and ultimately improving the quality of life in canine lymphoma patients.

Exportin 1 (XPO1) as a Therapeutic Target

In recent years, the nuclear export receptor Exportin 1 (XPO1), also known as Chromosome Region Maintenance 1 (CRM1) has emerged as a promising therapeutic target in both human and veterinary oncology [Azizian and Li, 2020; Sendino *et al.*, 2018]. XPO1 is a key mediator of nuclear-cytoplasmic transport and is responsible for exporting more than 200 cargo molecules, including various RNA species, tumor suppressor proteins (TSPs) and growth regulators, from the nucleus to the cytoplasm [Balasubramanian *et al.*, 2021; Parikh *et al.*, 2014]. These include critical TSPs such as p53, p21, p27, and members of FOXO family [Gravina *et al.*, 2014; Hill *et al.*, 2013; Özdaş and Canatar, 2021; Senapedis *et al.*, 2014].

Overexpression of XPO1 has been documented in a wide range of human and veterinary malignancies, including lymphoma, leukemia, osteosarcoma, and melanoma [Breitbach *et al.*, 2021; Breit *et al.*, 2014; London *et al.*, 2014]. In tumor cells, aberrant upregulation of XPO1 leads to cytoplasmic mislocalization and functional inactivation of key TSPs, thereby facilitating oncogenesis, tumor progression, and therapeutic resistance [Lapalombella *et al.*, 2012; Turner *et al.*, 2012]. Elevated XPO1 expression has been associated with poor prognosis, aggressive disease behavior, and shortened survival across multiple hematological malignancies and solid tumors [Benkova *et al.*, 2021; Nie *et al.*, 2022]. Comparable molecular alterations have been observed in canine cancers, suggesting that the oncogenic role of XPO1 is evolutionally conserved between species [Breit *et al.*, 2014; Breitbach *et al.*, 2021; Grayton *et al.*, 2017; London *et al.*, 2014]. These findings collectively highlight XPO1 as a critical regulator of tumor biology and a rational target for therapeutic intervention in both human and veterinary oncology.

Development of Selective Inhibitors of Nuclear Export (SINE)

The discovery of Selective Inhibitors of Nuclear Export (SINE) compounds has provided a novel therapeutic approach to target XPO1 [Benkova *et al.*, 2021; Talati and Sweet, 2018; Turner *et al.*, 2012]. These small molecules, including verdinexor (KPT-335), covalently bind to the Cys528 residue of XPO1, thereby preventing its interaction with cargo proteins [Balasubramanian *et al.*, 2021; Ou *et al.*, 2022; Pan *et al.*, 2021; Parikh *et al.*, 2014]. By preventing nuclear export, SINE compounds restore the nuclear localization and function of TSPs, leading to cell-cycle arrest, apoptosis, and suppression of tumor proliferation [Conforti *et al.*, 2017; Lapalombella *et al.*, 2012; Sendino *et al.*, 2018]. Preclinical studies have demonstrated that KPT-335 exerts potent anti-proliferative effects in various canine cancer cell lines, including lymphoma, osteosarcoma, and melanoma [Breitbach *et al.*, 2021; Breit *et al.*, 2014; London *et al.*, 2014]. Phase I and II clinical trials in dogs with naturally occurring cancers have shown that KPT-335 is orally bioavailable, well tolerated, and biologically active [London *et al.*, 2014; Sadowski *et al.*, 2018]. Notably, therapeutic responses have been observed in both B-cell and T-cell lymphomas, with particularly promising efficacy in T-cell subtypes, which are typically less responsive to conventional chemotherapy [Sadowski *et al.*, 2018]. These findings collectively underscore the clinical potential of XPO1 inhibition as a novel therapeutic avenue in canine oncology.

Rationale for Combination Therapy

While SINEs have demonstrated antitumor activity as single agents, combination therapy represents a rational and potentially superior strategy to enhance efficacy and overcome resistance to conventional chemotherapeutics [Galinski *et al.*, 2021; Kashyap *et al.*, 2016; Wang *et al.*, 2025]. In human oncology, XPO1 inhibitors have shown synergistic effects when administered in combination with standard cytotoxic agents such as doxorubicin and

vincristine, resulting in enhanced tumor suppression, increased apoptotic signaling, and improved overall treatment outcomes [Lewin *et al.*, 2021; Seymor *et al.*, 2021; Škubník *et al.*, 2021; Turner *et al.*, 2013]. Mechanistically, the inhibition of nuclear export by SINE compounds can restore nuclear retention and function of key tumor suppressors, thereby sensitizing cancer cells to DNA-damaging and mitotic-stress-inducing agents [Chakravarti *et al.*, 2024; Hill *et al.*, 2013; Wang *et al.*, 2019]. This complementary mechanism may potentiate cytotoxicity, delay or prevent the emergence of resistance, and achieve more durable therapeutic responses in canine malignancies. Preclinical studies in canine osteosarcoma models have demonstrated enhanced antiproliferative effects when SINE compounds are combined with chemotherapeutic agents [Breitbach *et al.*, 2021], suggesting that similar synergistic interactions may occur in canine lymphoma. Such combinatorial approaches are particularly appealing in the context of drug-resistant disease, where simultaneous targeting of parallel oncogenic pathways may restore chemosensitivity and extend the duration of clinical remission.

Objectives of the Study

Taken together, these insights highlight the dual importance of elucidating the biological role of XPO1 in canine lymphoma and assessing the therapeutic potential of its pharmacologic inhibition, either as a single agent or in combination with conventional chemotherapeutics. Accordingly, the present study aims to: (i) characterize the expression of XPO1 at both the mRNA and protein levels in a panel of canine lymphoma cell lines, and to compare these expression patterns with those observed in normal peripheral blood mononuclear cells (PBMC); (ii) evaluate the *in vitro* efficacy of KPT-335 as a single agent, with particular emphasis on its effects on cell proliferation and viability across distinct B-cell and T-cell lymphoma subtypes; and (iii) investigate the combinational potential of KPT-335

with standard chemotherapeutic agents, specifically doxorubicin and vincristine, and to determine whether such combination produce enhanced antiproliferative and cytotoxic effects relative to monotherapy.

Significance and Implications

By integrating molecular characterization with therapeutic evaluation, this study aims to clarify the role of XPO1 in canine lymphoma and to establish a preclinical rationale for incorporating XPO1 inhibitors into future therapeutic regimens. Beyond its relevance to veterinary oncology, the findings may also contribute to the broader field of comparative oncology by providing translational insights applicable to human lymphoma. Ultimately, this research seeks to advance the understanding of XPO1 biology, expand the therapeutic landscape for both canine and human lymphoma.

CHAPTER 1

Exportin 1 (XPO1) Expression and Effectiveness of XPO1 Inhibitor Against Canine Lymphoma Cell Lines

Abstract

Lymphoma is the most common neoplasm of lymphoid tissues in dogs. Exportin 1 (XPO1) is an important major nuclear receptor for exporting proteins and RNA species. The XPO1 upregulation can eliminate some TSPs function upon their nuclear-cytoplasmic export. The XPO1 inhibitor, KPT-335, blocks the translocation of TSPs and restore their function to inhibit cell proliferation, induce cell cycle arrest, and apoptosis. This *in vitro* study aimed to evaluate the XPO1 mRNA and protein expression in canine lymphoma cell lines and confirm the relevance with KPT-335. XPO1 mRNA and protein levels were quantified, and the effect of KPT-335 was assessed by a cell proliferation assay. The results indicated that XPO1 mRNA and protein were highly expressed in 17-71, CLBL-1, CLC, CLGL-90, and UL-1, and were moderately expressed in GL-1, Ema, and Nody-1. All canine lymphoma cell lines showed dose-dependent growth inhibition and decreased cell viability in response to KPT-335 which IC_{50} concentrations ranged from 89.8-418 nM. The expression levels of XPO1 mRNA and protein were related; however, no correlation was found between those expression levels and the efficacy of KPT-335. These findings suggest that XPO1 may represent a promising target for therapeutic intervention in canine lymphoma.

Keywords: canine lymphoma; cell lines; dogs; exportin 1; immunomodulation; *in vitro* study; protein transport; XPO1 inhibitor

INTRODUCTION

Lymphoma is a common and considerably aggressive hematologic malignancy that affects the lymphatic system, which is crucial to immune function [Sapierzyński *et al.*, 2016; Valli *et al.*, 2012]. The similarities between canine and human lymphoma include cellular morphology, clinical manifestations, pathology, tumor behavior, molecular biology investigations, genetic aberrations, diagnostic methods and therapeutic approaches [Avery, 2020; Ponce *et al.*, 2010; Sapierzyński *et al.*, 2016]. Canine lymphoma is characterized by the uncontrolled proliferation of lymphocytes, with multicentric lymphoma being the most prevalent, which leads to the formation of tumors in lymph nodes, spleen, liver, and other organs and presents with symptoms such as generalized lymphadenopathy, anorexia, loss of activity, and organ dysfunction [Ponce *et al.*, 2010; Valli *et al.*, 2012].

In canine lymphoma, systemic treatments, particularly multi-drug chemotherapy, are the most effective options, achieving sub-optimal response rates. Chemotherapy have significantly improved quality of life and longevity in many cases, however, most cases resulted in relapse and/or resistance to chemotherapy [London *et al.*, 2014; Valli *et al.*, 2012; Zandvliet, 2016]. Numerous studies focusing on targeted therapies and immunotherapies emerged, aiming to overcome the challenge of cancer treatments effectively [Turner *et al.*, 2012; Wang *et al.*, 2019]. The increasing number of comparative studies in various cancer research aims to enhance therapy efficiency, support current chemotherapy agents' work, and potentially substitute previous treatments with minimal side effects [Avery, 2020; Ponce *et al.*, 2010; Zandvliet, 2016]. Hence, there is an urgent need to identify novel therapy targets to improve the adverse outcomes of canine lymphoma patients.

In human oncology, the nuclear export receptor Exportin 1 (XPO1), also called Chromosome Region Maintenance 1 (CRM1), has gained attention as a promising therapeutic target due to its crucial role in transporting various proteins and RNA molecules from the

nucleus to the cytoplasm, thereby maintaining cellular homeostasis and regulating key signaling pathways [Sendino *et al.*, 2018; Talati and Sweet, 2018]. XPO1 overexpression in cancer cells can lead to the mislocalization of multiple TSPs including p21, p53, p73, p27, Forkhead box O (FOXO), Retinoblastoma (Rb)1, Adenomatous polyposis coli (APC), Breakpoint Cluster Region-Abelson (BCR-ABL), Inhibitor of kappa B (IκB) and Protein Phosphatase (PP)2A, and contributing to oncogenesis and cancer progression [Benkova *et al.*, 2021; Parikh *et al.*, 2014; Turner *et al.*, 2012].

Recent studies have highlighted the potential of XPO1 inhibitors, in targeting these pathways to induce apoptosis and inhibit tumor growth. Selective inhibitors of nuclear export (SINE) compounds, such as KPT-335 (verdinexor), have been developed to target XPO1 [Breitbach *et al.*, 2021; Breit *et al.*, 2014; Ou *et al.*, 2022; Pan *et al.*, 2021]. A phase I study demonstrated that KPT-335 is safe and exhibits biological activity in dogs with spontaneous cancers [London *et al.*, 2014]. The study also revealed potent activity of KPT-335 in one cell line each for canine diffuse large B cell lymphoma (DLBCL), mast cell tumor, melanoma and osteosarcoma, as well as clinical samples of canine DLBCL cultured with CD40L stimulation. However, the factors determining susceptibility to KPT-335 remain unclear.

Further, a phase II study involving fifty-eight dogs with naïve or relapsed B-cell and T-cell lymphoma reported that oral administration of KPT-335 was well tolerated, with mild anorexia being the most common side effect [Sadowski *et al.*, 2018]. Among the evaluable dogs, the objective response rate (ORR) was 37% (20/54), with approximately 30% in B-cell lymphomas and about 70% in T-cell lymphomas. Given that canine T-cell lymphomas typically have a poor prognosis and respond less effectively to conventional treatments compared to B-cell lymphomas [Avery, 2020; Valli *et al.*, 2012; Zandvliet, 2016], the observed efficacy of KPT-335 in T-cell lymphoma suggests its potential therapeutic utility. Therefore, this *in vitro* study aims to investigate whether the sensitivity to KPT-335 is related to XPO1 gene and/or

protein expression, and investigated potential differences in drug sensitivity between B-cell and T-cell lymphoma cell lines. First, I identified whether the XPO1 mRNA and protein levels were overexpressed and have a correlation. Then, I examined if the effectivity of the XPO1 inhibitor (KPT-335) was related to the XPO1 gene and protein expression in canine lymphoma cell lines. In the context of veterinary oncology, this study will provide auspicious implications for future research and clinical applications in canine lymphoma.

MATERIALS AND METHODS

1. Cell Lines

A total of eight established canine lymphoma cell lines were used in this study. B-cell lymphoma cell lines include 17-71, CLBL-1 and GL-1, whereas T-cell lymphoma cell lines are CLC, CLGL-90, Ema, Nody-1 and UL-1 (Table 1) [Maylina *et al.*, 2022]. A western blot analysis used a human lymphoblastoid cell line derived from Burkitt's lymphoma patient, Raji, as a positive control for XPO1 protein expression [Wang *et al.*, 2019]. Live cells with a 90% or above proportion were eligible to start the cell culture process. The R10 complete medium [RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan) and 55 μM 2-mercaptoethanol] was used to sustain all canine lymphoma cell lines. All cells were cultivated at 37 °C in a humidified incubator with 5% CO_2.

Peripheral blood mononuclear cells (PBMCs) were collected from healthy Beagle dogs that kept for blood donation from Yamaguchi University Animal Medical Center (YUAMEC) Japan, using density-gradient centrifugation method, and used as normal blood cells mainly containing lymphocytes and monocytes. Briefly, whole blood was drawn by jugular venipuncture into an EDTA tube. Whole blood was then placed in a 15 mL tube, centrifuged at 3,000 rpm for 15 minutes at room temperature (RT), and the resulting buffy coat fraction was transferred and mixed with PBS then overlaid onto Lymphoprep™ (Alere Technologies AS, Oslo, Norway) in a 15 mL tube for density-gradient centrifugation. The tube was centrifuged at 1,700 rpm for 20 minutes at RT. A mononuclear layer between the Lymphoprep™ and the supernatant was collected and transferred in a separate 15 mL tube pre-filled with 10 mL of PBS then centrifuged at 1,500 rpm for 10 minutes at RT. The supernatant was carefully aspirated, and the cell pellet was resuspended in 10 mL of PBS and re-centrifuged as in the previous step. The supernatant was discarded and the sediment was PBMCs and suspended

with R10 medium. PBMCs were counted using Turk's solution and set at a concentration of 4×10^6 live cells/mL for cell proliferation and viability assessment. After counting, re-centrifuged at 1,500 rpm for 10 minutes at RT, then aspirated the supernatant and resuspended with R10 medium with the desired volume after calculating the number of cells.

2. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from the canine lymphoma cell lines using a NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized from up to 0.5 μg of total RNA using ReverTra® Ace qPCR RT Master Mix with gDNA Remover kit (TOYOBO, Osaka, Japan). The NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the total quantity of RNA and cDNA. The absorbance waveform was utilized to determine the purity of the nucleic acid. The RNA and cDNA were frozen at -80 and -30ºC for later use, respectively. The primers utilized for amplification were ribosomal protein L32 (RPL32) as endogenous control (RPL32F and RPL32R; amplicon length 100 bp, Table 2) [Peters *et al.*, 2007], and XPO1 primers (XPO1F and XPO1R; amplicon length 143 bp, Table 2) which specifically self-designed based on canine sequence (XM_038680688).

According to the manufacturer's protocol, the cells' cDNA was mixed with XPO1 or RPL32 primers using THUNDERBIRD® Next SYBR® qPCR Mix (TOYOBO, Osaka, Japan). The qRT-PCR was performed to analyze mRNA expression levels using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the thermal cycler condition as follows: 95ºC for 30 seconds (initial denaturation); 95ºC for 5 seconds, 60ºC for 10 seconds and 72ºC for 10 seconds for 40 cycles (thermal cycling); and then performing melting curve analysis.

Expression levels were normalized to RPL32, which served as the endogenous control. RPL32 was stably expressed in my sample sets and accurate for normalization in canine gene expression studies [Peters *et al.*, 2007]. All reactions were performed in triplicate including no-template controls for each gene and evaluated in three independent experiments. Quantifications of XPO1 gene expression for all real-time PCR data were calculated using the comparative threshold cycle method (CT). The ΔCt was calculated by deducting the XPO1 CT value from the RPL32 CT value. The expression levels of XPO1 mRNA were determined as $2^{-\Delta Ct}$.

3. Western Blotting

All canine lymphoma cell lines were selected to assess the presence of XPO1 proteins. The whole cell lysates were lysed with NP40 lysis buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl,1 mM EDTA, 1% NP40] mixed with phosphatase and protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were subjected to BCA protein assay (Takara Bio, Shiga, Japan) to determine the protein concentration. Then, each protein sample was loaded into lanes in 6% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel to detect the XPO1 (6% gel) and β-actin (12% gel) protein.

Following the electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA) and blocked with 5% skimmed milk mixed with Tris-buffered saline containing 0.1% Tween-20 (TBST) before probing with mouse monoclonal anti-XPO1 (dilution 1:2000; C-1; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C on a rotary plate. To obtain an endogenous control, a mouse monoclonal anti-β-actin (dilution 1:5000; AC-15; Sigma-Aldrich, Saint Louis, MO, USA) diluted in 0.5% skimmed milk-TBST was used. After being washed three times with TBST, the membranes were probed with secondary labeling using goat anti-mouse IgG HRP (dilution 1:4000; Santa

Cruz Biotechnology, Dallas, TX, USA) and incubated for one hour at RT on a rotary plate. At the end of the incubation period, they were re-washed three times with TBST, then incubated for 5 minutes with SuperSignal™ West Pico PLUS Chemiluminescent Substrate reagent (Thermo Fisher Scientific, Waltham, MA, USA) and visualized using an AMERSHAM ImageQuant 800 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The quantification of the signal intensity of XPO1 protein expression was analyzed using ImageJ software version 1.54d (National Institutes of Health, Bethesda, MD, USA) and normalized with the intensity of β-actin.

4. Cell Proliferation Assay

To examine half-maximal inhibitory concentration (IC_{50}) of KPT-335, a Water-Soluble Tetrazolium salt (WST) assay was conducted using the cell counting kit-8 assay (CCK-8; Dojindo Laboratories, Kamimashiki, Japan). KPT-335 (Selleck Chemicals, Houston, USA), was dissolved in DMSO at a concentration of 50 mM. Eight canine lymphoma cell lines at a density of 2×10^5 to 8×10^5 cells/mL were treated with KPT-335 at various concentrations (0, 0.001, 0.01, 0.1, 1, 10 and 100 μM) in 96-well plates (triplicate) for 48 hours. Cell proliferation was assessed by adding 10 μL WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) and incubated for 4 h. The absorbance at 450 nm wavelength was measured using a Thermo Scientific™ Multiskan™ FC microplate reader. The cell proliferation was calculated using the ratio of the absorbance of treated cells to the absorbance of control cells, and the data were expressed as percentages. The IC_{50} of KPT-335 was calculated using Excel software (Microsoft) after plotting proliferation values on a logarithmic curve.

5. Trypan Blue Exclusion Assay

The canine lymphoma cell lines were seeded at densities ranging from 1×10^4 to 4×10^4 cells in 96-well plates. All groups of cells were treated with either 0.2% of DMSO, 0.125, 0.25, or 0.5 μM of KPT-335 for 48 hours. Each cell line was seeded in triplicate for each treatment group and the entire experiment was repeated three times. The KPT-335 treated cells were harvested after 48 hours of drug treatment. The trypan blue exclusion assay was performed to determine the number of viable cells present in a cell suspension. The cells were stained with trypan blue after 48 hours drug treatment and viable cells were counted with a hemocytometer. The percentage of viable cells was calculated with the total cell count.

6. Statistical Analyses

All statistical analyses were conducted by JMP® Pro software version 18.1.1 (SAS Institute, Tokyo, Japan). XPO1 mRNA and protein expression levels were analyzed using one-way ANOVA and the non-parametric Kruskal-Wallis test, respectively. Significance values were compared with the control using Dunnett's method. One-way ANOVA followed by Dunnett's method for comparisons with control was used for WST and trypan blue assay to compare with no-KPT treatment cells. The p-value < 0.05 was considered significant.

RESULTS

1. Overexpression of the XPO1 mRNA in canine lymphoma cell lines

I evaluated the XPO1 gene expression in the eight canine lymphoma cell lines, including three B-cell lines (17-71, CLBL-1 and GL-1) and five T-cell lines (CLC, CLGL-90, Ema, Nody-1 and UL-1) as well as PBMCs obtained from a healthy dog. The qRT-PCR analysis revealed that 17-71 and CLBL-1 from B-cell lines, as well as CLC, CLGL-90 and UL-1 from T-cell lines, showed a significant increase in XPO1 mRNA expression when compared to that of canine PBMCs as a normal cell control (Figure 1A). CLC and UL-1 from T-cell lines showed a significant increase in relative expression to the RPL32 gene with values of 0.1538 ± 0.033 and 0.0810 ± 0.033, respectively, and p-values < 0.01. Meanwhile, significant increases in relative expression of 17-71 and CLBL-1 from B cells and CLGL-90 from T cells were observed in sequence at 0.0520 ± 0.021, 0.0513 ± 0.013, and 0.0562 ± 0.014, respectively, with a p-values < 0.05. In contrast, there was no significant difference between GL-1, Ema or Nody-1 cell lines and the PBMCs. In total, five of eight cell lines showed significant overexpression of the XPO1 mRNA.

2. Overexpression of XPO1 protein in the canine lymphoma cell lines

Western blot analysis was conducted to investigate the expression of the XPO1 protein in the canine lymphoma cell lines and the PBMCs as normal control, as well as a Raji cell line as a positive control. The expression of XPO1 protein in the PBMCs was less expressed when compared to those of the canine lymphoma cell lines as shown in Figure 1B. In the B cell lines, only 17-71 showed significant higher XPO1 protein expression with a p-value < 0.05 compared to those of CLBL-1 and GL-1. While in the T cells, the expression of XPO1 protein in CLC and UL-1 were significantly high with p-values < 0.01, and CLGL-90 showed a significant expression with a p-value < 0.05. While CLBL-1, GL-1, Ema and Nody-1 had lower XPO1

protein expression compared to the other cell lines, and showed no significant difference compared to that of the PBMCs (Figures 1B, 1C).

Based on the expression results of the XPO1 gene and protein, it was summarized that XPO1 gene expressions in eight canine lymphoma cell lines were seemed to be generally higher than those in normal cells (PBMCs) and related to their XPO1 protein expressions, except for CLBL-1, where the XPO1 protein expression analysis yielded no significant difference. However, specific trends in neither XPO1 mRNA nor protein expressions were observed in B-cell lines and T-cell lines.

3. KPT-335 inhibits the proliferation of canine lymphoma cells

To examine an inhibitory effect of KPT-335, an XPO1 inhibitor, a WST assay was conducted using various concentrations of KPT-335 ranging from 0 to 100 μM, and the IC_{50} values of KPT-335 were calculated from eight canine lymphoma cell lines. As shown in Figure 2, there was a considerable reduction in cell proliferation depending on the concentration of KPT-335 with IC_{50} values ranging at the nanomolar level between 89.8 - 418 nM in B- and T-cell lines.

In the B-cells group, KPT-335 had IC_{50} values of 89.8 ± 2.07, 108.6 ± 30.54 and 294.3 ± 25.57 (nM) for 17-71, CLBL-1 and GL-1, respectively (Table 3). Meanwhile, in the T-cells group, CLC, CLGL-90, Ema, Nody-1 and UL-1 had IC_{50} values of 224.7 ± 78.38, 215 ± 50.96, 147.8 ± 49.69, 220.5 ± 27.34 and 418 ± 78.6 (nM), respectively (Table 3). In contrast, the PBMCs resulted in high IC_{50} value of 872.5 ± 66.72 nM which showed that PBMCs as normal cell was not sensitive to KPT-335 exposure (Table 3 and Figure 2C).

Next, I examined a cell viability to further assess the effect of KPT-335 on the canine lymphoma cell lines. The cell viability measured by a cell counting with trypan blue exclusion revealed a decrease in the number of viable canine lymphoma cells (Figure 3). Exposure to

0.125, 0.25 and 0.5 μM of KPT-335 reduced the cell viability significantly in all eight canine lymphoma cell lines compared to no-treated cells (0 μM). This finding was consistent with the results of the cell proliferation assay, which showed that KPT-335 inhibited cell proliferation in a dose-dependent manner. All the cell lines used in this study demonstrated a considerable decrease in the average percentage of living cells. At 0.5 μM concentration, all canine lymphoma cell lines exhibited a percentage of viable cells less than 50%. In contrast, the viability assay in PBMC was remained in high percentage, indicating similar result with cell proliferation assay that normal PBMCs were less sensitive to KPT-335 compared with canine lymphoma cell lines (Figure 3C).

DISCUSSION

In cancers, the mislocalization of many TSPs caused by the overexpression of the nuclear export protein, XPO1 contributed to cell proliferation and increased metastatic potential [Balasubramanian *et al.*, 2022; Sendino *et al.*, 2018]. Canine cancer studies reported the overexpression of XPO1 mRNA levels from patient-derived tissues and cell lines in osteosarcoma [Breitbach *et al.*, 2021]. Notably, while XPO1 protein expression has been previously reported in canine DLBCL cells, including the CLBL-1 cell line and cryopreserved tumor cells [London *et al.*, 2014]; as far as I know, my study is the first to report XPO1 mRNA expression in canine lymphoma cells.

The expression levels of XPO1 mRNA and protein were well correlated between canine lymphoma cell lines (Figure 1). In addition, the expression levels of XPO1 mRNA and protein in PBMCs obtained from a healthy dog were lower than those in canine lymphoma cell lines except that of mRNA in Nody-1. This is concordant with a study reported that the expression of XPO1 in both gene and protein in the majority of canine osteosarcoma cells were significantly higher compared with normal canine osteoblast cells [Breitbach *et al.*, 2021], and the XPO1 protein overexpression demonstrated potent activity necessary for cell survival in canine cancers [London *et al.*, 2014]. In addition, XPO1 was also overexpressed compared to PBMC, normal B and T cells at the protein as well as mRNA level in human lymphoma/leukemia studies [Abeykoon *et al.*, 2019; Lapalombella *et al.*, 2012; Nie *et al.*, 2022]. In those reports, the authors did not compare the expression levels of XPO1 between mRNA and protein. The overexpression of XPO1 also reported in other human cancers such as thymic epithelial tumors, malignant melanoma, osteosarcoma, glioma, ovarian, pancreatic, cervical, colorectal and gastric cancers [Aladhraei *et al.*, 2019; Conforti *et al.*, 2017; Sexton *et al.*, 2019; Turner *et al.*, 2012; Yang *et al.*, 2014]. My analysis showed that the XPO1 protein

expression tended to be high in all the canine lymphoma cell lines examined, suggesting that they could serve as potential therapeutic targets in canine lymphoma cells.

Studies in human hematologic malignancies have demonstrated alterations in the aberrant expression of XPO1 including its overexpression, deregulation or dysfunction. They showed several mechanisms such as amplification of the *XPO1* gene, chromosomal translocation with *XPO1* gene locus, and *XPO1* mutation as abnormalities [Camus *et al.*, 2017; Miloudi *et al.*, 2020; Nagasaka *et al.*, 2021]. Large-scale genomic analyses of human cancers identified mutational hotspots in the *XPO1* gene and also recurrent heterozygous *XPO1* mutations in a variety of cancer types, including chronic lymphocytic leukemia (CLL), Hodgkin's lymphoma and esophageal carcinoma [Taylor *et al.*, 2019]. These mutations resulted in the dysregulation of TSPs export, leading to tumorigenesis, aggravated disease progression and associated with poor survival outcomes [Nagasaka *et al.*, 2021; Walker *et al.*, 2021]. Additionally, studies in B-cell lymphoma and non-small cell lung cancer (NSCLC) indicated that *XPO1* mutations may result in resistance to conventional chemotherapy, however, cells with mutated *XPO1* demonstrated increased sensitivity to XPO1 inhibitors so that the therapeutic remained effective [Camus *et al.*, 2017; Miloudi *et al.*, 2020]. I conducted *XPO1* gene mutation analysis in the eight canine lymphoma cell lines and found that there were no mutations presented in the coding region of the canine *XPO1* gene (data not shown). Therefore, the cause of overexpression of XPO1 in the canine lymphoma cell lines observed in this study needs to be clarified in future studies.

Recently, studies using SINE compounds have been conducted in human hematologic malignancies. The efficacy of various types of SINE compounds was investigated in non-Hodgkin lymphoma (NHL), specifically T-cell lymphoma, Mantle cell lymphoma and DLBCL cell lines, which showed a significant decrease in cell proliferation at concentrations ranging in nanomolar concentration up to 500 nM [Abeykoon *et al.*, 2019; Nie *et al.*, 2022]. Some *in*

vitro and *in vivo* studies in other human cancers also revealed low IC_{50} in nanomolar concentrations of various SINE compounds, affected the nuclear retention of many TSPs, restore their function to induce cell cycle arrest and cell proliferation inhibition leading to apoptosis [Aladhraei *et al.*, 2019; Conforti *et al.*, 2017; Parikh *et al.*, 2014; Sexton *et al.*, 2019]. Similar to those studies, my study showed that KPT-335 is effective against canine lymphoma cell lines and exhibited a low IC_{50} range at concentrations of 89.8-418 nM (Figure 2 and Table 4). These low IC_{50} indicated that KPT-335 has a significant anti-proliferative effect against canine lymphoma cells. In canine cancers, KPT-335 or verdinexor, has been exhibited good biological activities in canine malignant melanoma, osteosarcoma, mammary and transitional cell carcinoma (TCC) cell lines *in vitro* [Breitbach *et al.*, 2021; Breit *et al.*, 2014; Grayton *et al.*, 2017]. Since the mean KPT-335 C_{max} was reported to be 278 ng/mL (0.628 μM) in the phase II canine lymphoma study [Sadowski *et al.*, 2018], my *in vitro* results at a concentration of 0.5 μM, close to the Cmax, support the therapeutic efficacy and tolerability of KPT-335 with manageable adverse effects in canine lymphoma patients.

Consistent with the effects of cell proliferation, KPT-335 exposure in escalating concentrations showed a cytotoxicity effect which significantly reduced cell viability in canine lymphoma cell lines (Figure 3). Similar findings have been reported in human CLL and gastric cancer studies, where exposure to SINEs has led to the nuclear retention of TSPs such as FOXO, IκB, and p53, leading to cell cycle arrest, reducing cell viability and promoting cell death [Grayton *et al.*, 2017; Lapalombella *et al.*, 2012; Sexton *et al.*, 2019]. Based on my findings, KPT-335 has shown cytotoxic effects in canine lymphoma cell lines which are characterized by decreased cell viability, however, the localization of TSPs in the nucleus which leads to cell cycle arrest and apoptosis has yet to be clarified.

Although the XPO1 gene and protein expression showed a significant correlation, my study found no correlation between the sensitivity to KPT-335 and the XPO1 mRNA and

protein expression in canine lymphoma cell lines. Similar to my results, a study using canine osteosarcoma cells reported that the expression level of XPO1 protein was not directly correlated with sensitivity to KPT-335 [Breitbach *et al.*, 2021]. Investigation in human hematologic tumor cell lines revealed that the capability of SINE compounds binding to XPO1 was similar, regardless of their sensitivity to the drugs [Crochiere *et al.*, 2017]. No correlation was observed between drug sensitivity and dose-dependent occupancy, suggested that sensitivity to KPT-335 may not depend solely on binding to XPO1, but also on XPO1's target molecules. The observed lack of correlation points to additional intracellular determinants, such as apoptotic threshold and basal cell cycle activity, may influence therapeutic efficacy independently of XPO1 expression levels. Rapidly proliferating cells exhibit an increased dependency on nuclear export mechanisms, making them more susceptible to XPO1 inhibition. In human cutaneous T-cell lymphoma, the downregulation of survivin, a key inhibitor of apoptosis, following XPO1 inhibition may lower the cellular threshold for apoptosis, enhancing the drug sensitivity. The study also observed cell cycle arrest at G1 following treatment, suggesting that basal activity influences response dynamics [Chakravarti *et al.*, 2024]. In canine lymphoma cells, inactivation of p16 (CDKN2A) and hyperphosphorylation of its target, pRb, have been reported [Fujiwara-Igarashi *et al.*, 2014; Maylina *et al.*, 2022; Modiano *et al.*, 2007]. Previous studies in my laboratory have shown that among the cell lines used in this study, CLBL-1, CLC, CLGL-90, Ema, Nody-1, and UL-1 exhibit hyperphosphorylation of pRb and/or inactivation of p16, whereas no aberrations in pRb or p16 were found in 17-71 and GL-1 [Maylina *et al.*, 2022]. Unfortunately, my results showed no significant difference in the IC_{50} values of KPT-335 between 17-71 and GL-1 and the other cell lines, suggesting that differences in molecular mechanisms beyond the p16-pRb pathway may influence sensitivity to KPT-335. Importantly, at least at this stage, the expression levels of XPO1 mRNA or protein do not appear to serve as reliable predictors of KPT-335 sensitivity in canine lymphoma cells.

An immunophenotyping study with canine lymphomas reported that 68.7% of canine lymphomas originate from B cells, while 31.3% are of T-cell origin [Avery, 2020], and the latter are less responsive to conventional treatments compared to B-cell lymphomas [Avery, 2020; Valli *et al.*, 2012; Zandvliet, 2016]. Based on the phase II study [Sadowski *et al.*, 2018], I initially hypothesized that KPT-335 would be particularly effective against T-cell lymphomas, leading to the initiation of this *in vitro* study. However, no difference was observed in sensitivity between B-cell and T-cell lines. Given the wide heterogeneity of lymphoma subtypes, interpretation of these results remains challenging. To clarify the differences in KPT-335 sensitivity be-tween B and T cells, it will be necessary to conduct clinical studies using a larger cohort over extended period.

Combination regimens incorporating XPO1 inhibitors with other anti-cancer agents have demonstrated synergistic potential across multiple human malignancies [Parikh *et al.*, 2014]. In human cancers, promising efficacy has been reported using XPO1 inhibitors in combination with standard chemotherapies, proteasome or tyrosine kinase inhibitors [Benkova *et al.*, 2021; Sendino *et al.*, 2018; Talati and Sweet, 2018; Turner *et al.*, 2012]. Especially, clinical trials using XPO1 inhibitors in hematologic malignancies are being investigated in combination with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) protocol, as well as targeted agents such as ibrutinib, or has been combined with corticosteroid and/or bortezomib [Balasubramanian *et al.*, 2022; Benkova *et al.*, 2021]. In canine oncology, an *in vitro* study of osteosarcoma cell lines revealed enhanced cytotoxicity upon co-treatment with KPT-335 and anthracycline, may produce synergistic therapeutic potential [Breitbach *et al.*, 2021]. Prior research involving SINE compounds in both human and canine malignancies, support the potential for combinatorial treatment utilizing KPT-335 and cytotoxic agents in clinical cases where resistance to conventional chemotherapy has

emerged. Therefore, further studies are warranted to evaluate the efficacy of combining XPO1 inhibitors with other anticancer agents in the treatment of canine lymphoma.

This study demonstrated that KPT-335 effectively inhibited cell growth and reduced cell viability. However, this study has some limitations. First, further investigations were needed to provide advanced information about the effects of post-treatment of KPT-335 against canine lymphoma cell lines and the potential mechanism of KPT-335 in nuclear-cytoplasmic translocations of cargo proteins. Subsequently, samples from clinical cases should be used in future studies to evaluate the biological activity and the effectiveness of KPT-335.

CHAPTER 2

Combination effect of Exportin 1 inhibitor (KPT-335) with conventional chemotherapies in canine lymphoma cell lines

Abstract

Canine lymphoma is a common hematopoietic malignancy with limited therapeutic options, particularly in cases of drug resistance. Exportin 1 (XPO1), a key nuclear export receptor, mediates translocation of multiple TSPs. Aberrant overexpression of XPO1 impairs TSP function by exporting them to the cytoplasm, contributing to oncogenesis. KPT-335, a selective XPO1 inhibitor, restores TSP activity by blocking nuclear export, thereby inducing cell cycle arrest and apoptosis. This study investigates the *in vitro* effects of KPT-335 alone and in combination with conventional chemotherapeutics, doxorubicin and vincristine, on four canine lymphoma cell lines including CLBL-1, GL-1, Nody-1 and UL-1. Western blot analysis revealed that KPT-335 treatment reduced XPO1 protein expression in a dose-dependent manner, with proteasome inhibition reversing this effect, suggesting proteasome-mediated degradation. Cell proliferation assays demonstrated that combination treatments significantly inhibited cell growth compared to single-agent therapies. Synergy was quantified using the Chou–Talalay method, and it was noted that GL-1 and UL-1 cells exhibited consistent synergy across all drug combinations, while CLBL-1 and Nody-1 showed variable responses. These findings suggest that KPT-335 enhances the efficacy of doxorubicin and vincristine through proteasome-dependent XPO1 degradation. The observed synergistic interactions support the potential integration of KPT-335 into existing chemotherapy protocols for canine lymphoma. Further *in vivo* studies and clinical trials are warranted to validate its therapeutic utility and optimize combination regimens.

Keywords: canine lymphoma, chemotherapy, combination treatment, SINE

INTRODUCTION

The development of new anti-cancer drugs is often complicated by the persistent problem of chemoresistance [Azizian and Li, 2020; Balasubramanian *et al.*, 2022; Metzger *et al.*, 2024]. Canine lymphoma cells frequently adapt to conventional therapies, diminishing their effectiveness over time and leading to relapse [Avery, 2020; Valli *et al.*, 2012]. As time goes by, the clinical demand for treating canine lymphoma is increasing, however, the outcome of most lymphoma patients in canine with routine therapies are dismal [Zandvliet, 2016]. Conventional multi-agent chemotherapy protocols, particularly the cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) regimen, often yield high initial response rates [Valli *et al.*, 2012], but disease relapse and the emergence of chemoresistance remain major clinical challenges and limiting long-term remission and survival [Kuruvilla *et al.*, 2017; Quintanal-Villalonga *et al.*, 2021; Wolf-Ringwall *et al.*, 2020]. These challenges underscore the need for novel therapeutic strategies that enhance efficacy and overcome resistance mechanisms.

In human oncology, elevated XPO1 expression correlates with poor prognosis in hematologic malignancies and numerous solid tumor malignancies [Özdaş and Canatar, 2021]. Overexpression of XPO1 has been implicated in the pathogenesis of various cancers, including lymphoma, by promoting the cytoplasmic mislocalization and functional suppression of key TSPs [Gravina *et al.*, 2014]. SINE compounds, target this pathway and have shown antitumor activity in human oncology [Benkova *et al.*, 2021; Parikh *et al.*, 2014; Turner *et al.*, 2014]. KPT-335 (verdinexor) as one of SINE compound has demonstrated potent antitumor activity by restoring nuclear localization of TSPs, thereby inducing apoptosis, arresting cell cycle progression, and inhibiting oncogenic signaling pathways including PI3K/AKT and c-Myc/FOSL1, with evidence of tumor growth suppression and enhanced TSP retention [Ou *et al.*, 2022; Pan *et al.*, 2021]. In veterinary oncology, KPT-335 received conditional approval by

U.S. Food and Drug Administration (FDA) for canine lymphoma treatment in 2021 following Phase I and II trials in B-cell and T-cell non-Hodgkin lymphoma [London *et al.*, 2014; Sadowski *et al.*, 2018]. These findings support the therapeutic potential of XPO1 inhibition across diverse malignancies.

The long-period and costly cancer treatments, together with the challenge of chemoresistance, has driven interest in alternative therapeutic approaches such as combination therapy [Kashyap *et al.*, 2016; Rovsing *et al.*, 2023; Yang and Yuzbasiyan-Gurkan, 2022]. Human studies have reported enhanced antitumor effects when XPO1 inhibitors are combined with standard chemotherapies, resulting in increased nuclear TSP retention, reduced cell viability, and apoptosis induction [Chao *et al.*, 2015; Galinski *et al.*, 2021; Turner *et al.*, 2013; Wang *et al.*, 2025]. Notably, the synergistic interaction with doxorubicin has been observed in canine osteosarcoma reduced cell proliferation, suggesting a combinatorial benefit [Breitbach *et al.*, 2021]. Although Phase I and Phase II studies of KPT-335 have been reported [London *et al.*, 2014; Sadowski *et al.*, 2018], and its efficacy has been demonstrated in canine epitheliotropic cutaneous lymphoma [Vlodaver *et al.*, 2024], further studies in other lymphoma subtypes are lacking, and, to the best of my knowledge, no reports are available regarding its combination with CHOP chemotherapy in canine lymphoma. Taken together, these findings support for further exploration into SINE compounds as adjuncts to conventional chemotherapy in canine lymphoma.

Given this evidence, this chapter 2 investigates the combinatorial effects of KPT-335 with two widely used chemotherapeutic agents, doxorubicin and vincristine, on canine lymphoma cell lines *in vitro*. I hypothesize that the combination therapy of KPT-335 with either doxorubicin or vincristine will produce a synergistic cytotoxic effect, surpassing the efficacy of single-agent treatments. This study aims to inform future therapeutic approaches and support

the integration of targeted agents into existing chemotherapy protocols as a potential strategy to improve therapeutic outcomes in canine lymphoma.

MATERIALS AND METHODS

1. Cell lines

Four canine lymphoma cell lines were used in this study: CLBL-1 and GL-1 (B-cell lymphoma), and Nody-1 and UL-1 (T-cell lymphoma) [Primarizky *et al.*, 2025]. Cells with ≥90% viability were used for culture initiation. Cultures were maintained in R10 complete medium [RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal calf serum (FCS), 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan) and 55 μM 2-mercaptoethanol] at 37°C in a humidified atmosphere containing 5% CO_2.

2. Chemical compounds

Two conventional chemotherapy drugs, doxorubicin and vincristine, and one XPO1 inhibitor, KPT-335, were used in this study. KPT-335, an inhibitor of nuclear export transporter, was purchased in powder form (Selleck Chemicals, Houston, TX, USA) and dissolved in DMSO to prepare a stock solution at a concentration of 50 mM. Doxorubicin was purchased from Selleck Chemicals (Houston, TX, USA) in powder form, dissolved to prepare a stock solution at a concentration 50 mM in DMSO. Vincristine sulfate was purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan) in powder form, dissolved to prepare a stock solution at a concentration 20 mM in ddH_2O. MG132, a proteasome inhibitor, was purchased in powder form (FUJIFILM Wako Pure Chemical, Osaka, Japan) and dissolved in DMSO to prepare a stock solution at a concentration of 10 mM. All drugs were stored in the freezer at -80°C.

3. Western blot analysis

To investigate the effects of KPT-335 on XPO1 protein expression, cells were treated with DMSO (vehicle control), 0.1 μM or 1 μM KPT-335 (Selleck Chemicals) for 24 hours as described in previous study [Breitbach *et al.*, 2021]. All cell lines were analyzed for protein expression of XPO1, p16 and p53. Briefly, whole cell lysates were prepared using NP40 lysis buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl,1 mM EDTA, 1% NP40] supplemented with phosphatase and protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were determined using the BCA assay (Takara Bio, Shiga, Japan). Equal amounts of protein were resolved on 6% or 12% SDS-PAGE gels, transferred to PVDF membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBST).

Membranes were then incubated overnight at 4°C with rabbit monoclonal antibodies against XPO1 (dilution 1:4000; D6V7N; Cell Signaling Technology®, Danvers, MA, USA), mouse monoclonal anti-p16 (dilution 1:500; F-8; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-p53 (1:1000; 80/p53; BD Transduction Laboratories™, Lexington, KY) overnight at 4 °C on a rotary plate. β-actin was used as a loading control (dilution 1:5000; AC-15; Sigma-Aldrich, Saint Louis, MO, USA) diluted in 0.5% skimmed milk-TBST. All of antibodies used in this study are summarized in the Table 4. Then, the membranes were incubated for one hour at room temperature with HRP-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) on a rotary plate. Following another round of TBST washes, membranes were treated with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and visualized using the AMERSHAM ImageQuant 800 system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Band signal intensities of XPO1, p16 and p53 were quantified using

ImageJ software version 1.54d (National Institutes of Health, Bethesda, MD, USA) and normalized with the intensity of β-actin.

4. Cell proliferation assay

Cell proliferation following drug treatment was assessed using the Water-Soluble Tetrazolium salt (WST) assay. KPT-335 concentrations were selected based on approximate IC_{50} values from my previous study [Primarizky *et al.*, 2025], while those for doxorubicin and vincristine were determined through preliminary experiments. The Cell Counting Kit-8 assay (CCK-8; Dojindo Laboratories, Kamimashiki, Japan) was used to assess cell proliferation. Four canine lymphoma cell lines were seeded in 96-well plates in triplicates at a density of 1×10^4 to 4×10^4 cells per well in 50 µL of medium and incubated for 48 hours with 50 µL of drug solutions (control, KPT-335, doxorubicin, vincristine), either individually or in combination. All WST experiments included wells with only culture medium as the cell-free control (media blanks). After incubation, 10 µL of highly water-soluble tetrazolium salt, WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) was added to the cell and plates were incubated for 4 hours. Absorbance at 450 nm was measured using a Thermo Scientific™ Multiskan™ FC microplate reader. Absorbance values were directly proportional to viable cell numbers. Data were normalized to control and expressed relative cell proliferation values as percentages using Excel and produced cell proliferation curves.

5. Combination index (Chou-Talalay Method)

To assess the potential drug interactions, the enhanced cytotoxicity of KPT-335 was combined with either doxorubicin or vincristine based on approximate IC_{50} and the half of IC_{50} values, and drug interaction was analyzed using the Chou-Talalay method. Cell proliferation

was assessed via WST assay and OD 450 nm values were converted to fraction affected (Fa) values, representing the proportion of cells killed at a specific drug concentration. Fa values, corresponding drug doses (for single and combination treatments), and calculation of combination index (CI) values were analyzed using CompuSyn software version 1 (CompuSyn Inc. Paramus, NJ, USA). CI values < 1 indicate synergy; CI = 1 indicates additive effects, and CI > 1 indicates antagonism.

6. Statistical analyses

All experiments were performed in triplicate or at least two different times experiment independently. Statistical analyses were conducted by JMP® Pro software version 18.1.1 (SAS Institute, Tokyo, Japan). The data were analyzed using one-way ANOVA followed by Dunnett's *post hoc* multiple comparison test to compare the protein expressions with control. A p-value < 0.05 was considered statistically significant.

RESULTS

1. KPT-335 reduces XPO1 protein expression in canine lymphoma cell lines

Previous studies using human cancer cell lines and canine melanoma cell lines have shown that treatment with XPO1 inhibitors can result in XPO1 protein loss [Breit *et al.*, 2014; Hill *et al.*, 2013; Talati and Sweet, 2018]. To investigate whether canine lymphoma cells respond similarly, four cell lines (CLBL-1, GL-1, Nody-1 and UL-1) were treated with DMSO (vehicle control), 0.1 μM KPT-335, or 1 μM KPT-335 for 24 hours. XPO1 protein expression was assessed by Western blotting (Figure 4A). Consistent with prior findings, KPT-335 treatment reduced XPO1 protein expression across all canine lymphoma cell lines. Quantitative analysis revealed a statistically significant decrease in XPO1 expression of GL-1 and Nody-1 cells treated with 1 μM KPT-335 compared with vehicle-treated controls. In contrast, no significant changes were observed in XPO1 protein levels in the CLBL-1 and UL-1 cell lines, although both showed a decreasing trend (Figure 4B).

At both 0.1 μM and 1 μM concentrations, XPO1 expression was visibly reduced in all four cell lines compared to DMSO-treated controls. The reduction was more pronounced at 1 μM, while the 0.1 μM treatment led to a modest decrease. To confirm this mechanism in canine lymphoma cells, I co-treated cells with 5 μM MG132, a proteasome inhibitor, and 1 μM KPT-335 for 6 hours. The results demonstrated that MG132 reversed the KPT-335-induced reduction in XPO1 protein levels. Specifically, cells treated with KPT-335 alone exhibited decreased XPO1 expression, whereas co-treatment with MG132 preserved XPO1 levels, supporting the hypothesis that KPT-335 promotes proteasome-dependent degradation of XPO1 (Figure 5).

2. Combinatorial treatment with KPT-335 and chemotherapies inhibits cell proliferation in canine lymphoma cell lines

To evaluate the effect of combination therapy, approximate IC_{50} and half of IC_{50} concentrations of KPT-335 were selected based on a chapter 1 [Primarizky *et al.*, 2025], while those for doxorubicin and vincristine were determined through preliminary experiments. All four canine lymphoma cell lines were treated for 48 hours, with varying concentrations of KPT-335, doxorubicin, or vincristine, either alone or in combination. Analysis of cell proliferation curves revealed that doxorubicin and vincristine, when used in combination with KPT-335, more pronounced reduction in cell proliferation in B-cell lymphoma lines (CLBL-1 and GL-1) and T-cell lymphoma lines (Nody-1 and UL-1) compared with single-agent therapy (Figure 6). Moreover, in T-cell lymphoma lines (Nody-1 and UL-1), KPT-335 alone demonstrated superior growth inhibition relative to either chemotherapeutic agent. These findings suggest a synergistic or additive effect of KPT-335 when combined with conventional chemotherapies.

3. Synergistic effects of KPT-335 combined with doxorubicin or vincristine

Following 48-hour treatments with combinations of KPT-335 and either doxorubicin or vincristine, cell proliferation percentages for CLBL-1, GL-1, Nody-1, and UL-1 were calculated and analyzed using the Chou–Talalay method. Combination index (CI) values were derived to assess drug interactions, with $CI < 1$ indicating synergy, $CI = 1$ indicating additive effects, and $CI > 1$ indicating antagonism (Table 5). In CLBL-1 cells, KPT-335 generally exhibited synergistic effects when combined with doxorubicin ($CI < 1$), except at the approximate half-IC_{50} combination, where antagonism was observed ($CI = 1.318$). In contrast, all combinations of KPT-335 and vincristine demonstrated synergistic effects. In GL-1 cells, KPT-335 was synergistic with both doxorubicin and vincristine across all tested concentrations (approximate IC_{50} and half of IC_{50} values). In Nody-1 cells, additive and antagonistic

interactions were observed at the approximate half of IC_{50} combinations with CI = 1.095 for KPT-335 with doxorubicin and CI = 1.147 for KPT-335 with vincristine. However, combinations at approximate IC_{50} concentrations yielded synergistic effects. In UL-1 cells, similar to GL-1, all combinations of KPT-335 with either doxorubicin or vincristine resulted in synergistic effects at both approximate IC_{50} and the half of IC_{50} concentrations. Overall, the interactions were predominantly synergistic. However, certain combinations, particularly at sub-IC_{50} concentrations, exhibited additive or even antagonistic effects. These findings suggest that the therapeutic impact of KPT-335 may be context dependent, and drug ratios as well as cell type specific factors should be carefully considered when designing combination regimens.

CI values were plotted against the fraction affected (Fa) to assess drug interactions across different levels of cell growth inhibition. In GL-1 and UL-1 cells, all combinations of KPT-335 with doxorubicin or vincristine consistently demonstrated synergy (Figure 7B and 7D). In contrast, CLBL-1 and Nody-1 cells exhibited occasional antagonism (Figure 7A and 7C). Marked synergistic effects were observed at Fa > 0.5, an encouraging result, as stronger synergy at higher Fa values is considered more desirable for clinical translation. At Fa = 0.5, Nody-1 cells showed antagonism with KPT-335 and vincristine; however, synergy emerged at higher Fa values. Interestingly, GL-1 cells demonstrated increasing CI values with rising Fa, transitioning from synergy to an additive effect (CI = 1) at Fa = 0.5. These findings underscore the importance of evaluating drug interactions across a range of Fa values. As higher Fa values are more clinically relevant, the observed improvement in synergy at elevated Fa levels supports the potential therapeutic benefit of combining KPT-335 with conventional chemotherapies. Notably, CI values remained consistently below 1 in GL-1 and UL-1 cells across all levels of cell growth inhibition, indicating robust synergistic effects when KPT-335 was combined with doxorubicin or vincristine.

4. Expression of p53 and p16 tumor suppressors following KPT-335 treatment in canine lymphoma cell lines

Major TSPs such as p53 and p16 are play critical roles in regulating cell cycle arrest and apoptosis in response to oncogenic stress. To investigate whether KPT-335 modulates the expression of these proteins, Western blot analysis was performed on four canine lymphoma cell lines following 48-hour treatment with KPT-335 (Figure 8). The results revealed that p16 protein expression was absent in all four cell lines after KPT-335 exposure, regardless of concentration. A modest increase in p53 expression was observed in GL-1 cells at the highest concentration of KPT-335, suggesting a potential activation of p53-mediated pathways. In contrast, UL-1 cells maintained prominent and stable p53 expression levels, indicating limited responsiveness to KPT-335 in terms of p53 upregulation. CLBL-1 and Nody-1 cells exhibited no detectable p53 expression under any treatment condition.

DISCUSSION

This chapter investigated the effects of the SINE compound, KPT-335, both as a monotherapy and in combination with conventional chemotherapeutic agents, enhances the cytotoxic effects of doxorubicin and vincristine in canine lymphoma cell lines. The results demonstrated that KPT-335 reduced XPO1 protein expression in a subset of cell lines, inhibits cell proliferation, and enhances the cytotoxic effects of doxorubicin and vincristine through synergistic interactions.

My findings aligned with previous studies in both veterinary and human oncology. Reports in canine melanoma and osteosarcoma models have shown similar downregulation of XPO1 protein following KPT-335 treatment, accompanied by compensatory upregulation of XPO1 mRNA [Breit *et al.*, 2014; Breitbach *et al.*, 2021]. Consistent with those studies, KPT-335 treatment led to a reduction in XPO1 protein levels in dose-dependent manner, particularly in GL-1 and Nody-1 cells. In human cancers, SINE compounds have demonstrated suppression of oncogenic signaling and enhancement of tumor suppressor function [Azizian and Li, 2020; Özdaş and Canatar, 2022; Turner *et al.*, 2014]. KPT-335 suppressed esophageal cancer cell proliferation and migration through modulation of the XPO1/c-Myc/FOSL1 signaling pathway, and induced apoptosis by p53 nuclear accumulation in neuroblastoma [Ou *et al.*, 2022; Pan *et al.*, 2021]. Further investigation into downstream signaling and gene expression changes may elucidate the molecular mechanisms underlying these effects.

To confirm the role of proteasomal degradation in XPO1 suppression, I co-treated canine lymphoma cells with 5 μM MG132, a proteasome inhibitor, and 1 μM KPT-335, and incubated for 6 hours. Previous *in vitro* studies have shown that proteasome inhibitors can reverse the effect of SINE compounds, indicating that XPO1 inhibition leads to proteasome-dependent degradation, which substantially contributes to their antitumor activity [Gravina *et al.*, 2014]. Consistent with these findings, my results demonstrated that MG132 reversed the

KPT-335-induced reduction in XPO1 protein levels, supporting the hypothesis that KPT-335 promotes proteasome-dependent degradation of XPO1, a mechanism previously described in human multiple myeloma cells [Turner *et al.*, 2013; Wang *et al.*, 2025]. These results suggested that SINE compounds disrupt nuclear export by targeting XPO1, thereby sensitizing cancer cells to apoptosis and growth arrest.

Given the challenges of acquired drug resistance and chemotherapy-associated toxicity, combining drugs with non-overlapping mechanisms of action is increasingly emphasized [Galinski *et al.*, 2021; Lewin *et al.*, 2021; Rovsing *et al.*, 2023; Škubník *et al.*, 2021; Yang and Yuzbasiyan-Gurkan, 2022]. In this study, my findings demonstrated that KPT-335 enhances the cytotoxic effects of frontline chemotherapeutics doxorubicin and vincristine. While single-agent treatments showed significant inhibition of cell proliferation, combination therapy resulted in enhanced growth suppression, consistent with synergistic interactions. Similar synergism has been reported in human multiple myeloma cells treated with KPT-335 and doxorubicin [Turner *et al.*, 2013]. Mechanistically, XPO1 inhibition may cooperate with vincristine through multiple pathways: nuclear retention of TSPs, disruption of mitosis, enhanced cell cycle arrest via upregulation of p21/p27, reinforcement of vincristine-induced G2/M arrest, and modulation of drug efflux regulators and immune checkpoints [Azizian and Li, 2020; Fisher *et al.*, 2025; Škubník *et al.*, 2021]. Collectively, my data support the therapeutic potential of combining KPT-335 with standard chemotherapies, either doxorubicin or vincristine, significantly reduced cell proliferation across all four canine lymphoma cell lines. Moreover, the synergistic interactions observed in this study reinforce the potential of integrating targeted nuclear export inhibitors into existing chemotherapy protocols.

The four cell lines (CLBL-1, GL-1, Nody-1, UL-1) exhibited predominantly synergistic at the approximate IC_{50} doses combination. However, certain combinations, particularly at sub-IC_{50} concentrations, exhibited additive or even antagonistic effects. Effective combinations

were defined by enhanced efficacy relative to single-agent treatments [Breitbach *et al.*, 2021; Chao *et al.*, 2015; Galinski *et al.*, 2021]. These findings suggest that the therapeutic impact of KPT-335 may be context dependent, and drug ratios as well as cell type specific factors should be carefully considered when designing combination regimens.

CI vs. Fa plots revealed predominantly synergistic interactions, particularly at higher Fa values, which are more clinically relevant. In this study, the CI values were consistently < 1 in GL-1 and UL-1, indicating robust and reproducible synergy between KPT-335 and conventional chemotherapies. When synergistic effects (CI < 1) are observed at Fa > 0.5, they are generally considered more clinically relevant because they indicate greater cell kill [Chou, 2010]. Several studies have explored combinations of doxorubicin known to act synergistically with diverse anticancer agents in osteosarcoma, pancreatic and breast cancers [Das *et al.*, 2014; Mehraj *et al.*, 2022; Yang and Yuzbasiyan-Gurkan, 2022], and it showed consistent synergy with KPT-335 in my study. In contrast, data on vincristine combined with XPO1 inhibitors remain limited, even though my findings demonstrated clear synergism. Thus, both conventional chemotherapies in this study, determined to have synergistic effects with KPT-335 and have been found in multiple previous studies to act synergistically with different types of compounds including anti-cancer drugs and natural compounds [Chao *et al.*, 2015; Seymor *et al.*, 2021]. These results emphasize the importance of evaluating drug interactions across a range of Fa values and support the therapeutic potential of combining KPT-335 with conventional chemotherapies, especially at higher effect levels.

In this study, I evaluated the combinatorial effects of KPT-335 in combination with doxorubicin or vincristine over a broad concentration range. When KPT-335 was administered orally at 1.5 mg/kg (n = 4) or 1.25 mg/kg (n=4) three times per week, the mean C_{max} reached approximately 630 nM (278 ng/mL) [Sadowski *et al.*, 2018]. Immediately after intravenous administration, reported plasma concentrations were approximately 450 nM (245 ng/mL) for

doxorubicin (30 mg/m²) and 130 nM (119 ng/mL) for vincristine (0.7 mg/m²) [Hantrakul *et al.*, 2014; Wittenburg *et al.*, 2019]. Notably, all of these concentrations exceed those tested in my *in vitro* assays, indicating that the concentration range used in this study is clinically achievable.

I also investigated the impact of KPT-335 on the expression of key TSPs, p53 and p16. My study revealed modest upregulation of p53 in GL-1 and maintained p53 protein expression in UL-1 cells following KPT-335 treatment. XPO1 inhibition by selinexor (KPT-330) has been reported to induce autophagy-dependent apoptosis through the p53/mTOR pathway in gallbladder cancer [Zhao *et al.*, 2022], suggesting that a comparable mechanism may also occur in canine lymphoma cells. In addition, CLBL-1 and Nody-1 showed no detectable p53 expression under any treatment condition. Overall, my data suggest that KPT-335 may not uniformly restore p53 or enhance p53 function across all lymphoma.

In contrast, p16 expression remained undetectable in all cell lines after KPT-335 treatment. This lack of induction suggests that p16 is less responsive to XPO1 inhibition in canine lymphoma cells, as DNA methylation is known to contribute to p16 silencing in many canine lymphoma cells, including CLBL-1, Nody-1, and UL-1 cells [Maylina *et al.*, 2022]. Taken together, these findings indicate that KPT-335 may selectively influence p53 expression in certain canine lymphoma cell lines, while p16 remains unaffected. The differential modulation of p53 underscores the heterogeneity of canine lymphoma and highlights the importance of cellular context in determining therapeutic efficacy. Since the principal effect of XPO1 inhibition is to alter subcellular localization rather than total protein abundance, future studies assessing nuclear versus cytoplasmic distribution as well as downstream functional consequences will be necessary.

Taken together, my results support the continued evaluation of KPT-335 as a potential adjunct to conventional chemotherapy. Despite the promising *in vitro* findings, this study has several limitations. Further validation in animal models is essential to assess the therapeutic

efficacy, pharmacokinetics, and safety profile of KPT-335 in combination with conventional chemotherapies. Additionally, clinical trials in canine lymphoma patients will be essential to establish translational potential and to identify responsive subtypes.

GENERAL CONCLUSION

Lymphoma is one of the most common types of blood malignancies in dogs [Vail *et al.*, 2013; Zandvliet, 2016]. Cancer cells often exploit Exportin 1 (XPO1) to transport key tumor-suppressive proteins out of the nucleus [Azizian and Li, 2020], thereby abrogating their function [Dickmanns *et al.*, 2015; Gravina *et al.*, 2014]. XPO1 inhibitors disrupt the translocation, restore their function to inhibit cancer growth, induce cell-cycle arrest, and cause cancer-cell death [Balasubramanian *et al.*, 2022; Chao *et al.*, 2015; Galinski *et al.*, 2021; Kashyap *et al.*, 2016]. This thesis demonstrates the biological activity of the XPO1 inhibitor, KPT-335 (verdinexor), against canine lymphoma cell lines, and shows that its combination with two conventional chemotherapies, doxorubicin and vincristine which represents a promising therapeutic target in canine lymphoma.

In chapter 1, I investigated the expression and biological relevance of XPO1 in canine lymphoma cell lines. Quantitative real-time PCR and Western blotting analyses revealed elevated XPO1 mRNA and protein expressions in all eight canine lymphoma cell lines relative to normal cells (PBMCs). Cell-proliferation and viability assays showed that KPT-335 exert potent growth-inhibitory and cytotoxic effects at nanomolar concentrations. Although XPO1 expression was consistently higher in lymphoma cells, no clear correlation between expression level and drug sensitivity was observed, suggesting that KPT-335 activity is not solely dependent on baseline XPO1 abundance. These results collectively support XPO1 as a viable therapeutic target in canine lymphoma. Given the frequent development of drug resistance to standard chemotherapy in clinical cases, further investigation into combination strategies is warranted.

In chapter 2, I evaluated KPT-335 in combination with two conventional chemotherapies, doxorubicin and vincristine. KPT-335 not only demonstrated strong single-agent antitumor effects—likely through proteasome-dependent degradation of XPO1—but also significantly potentiated the cytotoxicity of both chemotherapeutic agents. Synergistic

interactions were observed, resulting in reduced proliferation and enhanced cell death. Such combinations may enable dose reductions of conventional agents, thereby potentially mitigating toxicity while improving treatment outcomes. These findings highlight the promise of KPT-335 in overcoming resistance to standard therapies and underscore the need for further *in vivo* studies to assess pharmacokinetics, safety, and efficacy in canine models. Clinical trials will be essential to validate therapeutic utility and to optimize combination regimens. Notably, because KPT-335 has activity in both veterinary and human oncology, these results also support broader translational relevance.

In conclusion, this study underscores the therapeutic potential of targeting XPO1 with KPT-335, both as a monotherapy and in combination with established chemotherapeutic agents. By advancing my understanding of XPO1 biology and elucidating mechanisms of drug synergy, this dissertation contributes to a foundation for developing more effective, less toxic, and resistance-overcoming treatment strategies for lymphoma in dogs, with potential relevance to human oncology.

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TABLES

Table 1. Characteristics of the eight canine lymphoma/leukemia cell lines published

Cell line	**17-71**	**CLBL-1**	**GL-1**	**CLC**	**CLGL-90**	**Ema**	**Nody-1**	**UL-1**
Classification/ diagnosis	Acute B-cell lymphoma	Multicentric B-cell lymphoma	Acute B-cell leukemia	Gastrointestinal T-cell lymphoma	Chronic large granular lymphocytic T-cell leukemia	Mediastinal T-cell lymphoma	Alimentary T-cell lymphoma	Renal T-cell lymphoma
Immunophenotype	CD1c+, CD11a+, CD11c+, CD18+, CD45+, CD45RA+, CD54+, CD49d+, CD79a+, CD34−, CD3−, TCRαβ−, TCRγδ−, CD21−	CD3−, CD4−, CD5−, CD8−, CD11a+, CD11d−, CD14−, CD21−, CD34−, CD45+, CD45RA+, CD56−, CD79αcy+, MHCII+, TCRγδ−, Thy-1−	CD3−, weak CD4+, CD5−, variably CD8+, CD11a+, weak CD11d+, variably CD14+, CD21−, CD34−, CD45+, CD45RA+, CD56−, CD79αcy+, MHCII−, TCRγδ−, Thy-1−	CD3−, CD4−, CD8α−, CD11a−, CD11b−, CD11c−, CD14−, CD18+, CD21−, CD34−, CD45+, CD45RA+, CD90−, TCRαβ−, TCRγδ−, MHCII+, IgM−, IgG−	CD45R, CD18+, CD3+, TCRαβ+, variably CD8α+, CD79a−	CD3+, CD4−, CD8α−, CD11a−, CD11b−, CD11c−, CD14−, CD18+, CD21−, CD34−, CD45+, CD45RA+, CD90+, TCRαβ−, TCRγδ+, MHCII−, IgM−, IgG−	CD3+, CD4−, CD8α−, CD11a−, CD11b−, CD11c−, CD14−, CD18+, CD21−, CD34−, CD45+, CD45RA+, CD90−, TCRαβ−, TCRγδ−, MHCII+, IgM−, IgG−	CD3−, CD4−, CD8α+, CD11a−, CD11b−, CD11c−, CD14−, CD18−, CD21−, CD34−, CD45+, CD45RA+, CD90−, TCRαβ−, TCRγδ−, MHCII−, IgM−, IgG−
PARR	ND	TCRγ−, IgH+	TCRγ+, IgH−	TCRγ−, IgH−	ND	TCRγ+, IgH−	TCRγ+, IgH−	TCRγ+, IgH−
p53/NFκB protein expression	ND	+	+	ND	ND	−	−	−
p16 protein expression	+	−	+	−	−	−	−	−
Phospho-pRb protein expression	−	+	−	+	+	+	+	+
ABCB1 (*MDR1*) gene expression	ND	Low	Low	ND	ND	high	ND	high
Initial description or publication	[Maylina *et al.*, 2022; Seiser *et al.*, 2011; Steplewski *et al.*, 1987; Suter *et al.*, 2005]	[Bonnefont-Rebeix *et al.*, 2016; Kojima *et al.*, 2017; Maylina *et al.*, 2022; Pawlak *et al.*, 2017; Rütgen *et al.*, 2010; Tomiyasu *et al.*, 2014]	[Bonnefont-Rebeix *et al.*, 2016; Kojima *et al.*, 2017; Maylina *et al.*, 2022; Nakaichi *et al.*, 1996; Pawlak *et al.*, 2017; Rütgen *et al.*, 2010; Seiser *et al.*, 2011; Tomiyasu *et al.*, 2014]	[Bonnefont-Rebeix *et al.*, 2016; Maylina *et al.*, 2022; Umeki *et al.*, 2013]	[Maylina *et al.*, 2022; Seiser *et al.*, 2011; Suter *et al.*, 2005]	[Bonnefont-Rebeix *et al.*, 2016; Kojima *et al.*, 2017; Maylina *et al.*, 2022; Tomiyasu *et al.*, 2014; Umeki *et al.*, 2013]	[Bonnefont-Rebeix *et al.*, 2016; Kojima *et al.*, 2017; Maylina *et al.*, 2022; Umeki *et al.*, 2013]	[Bonnefont-Rebeix *et al.*, 2016; Kojima *et al.*, 2017; Maylina *et al.*, 2022; Tomiyasu *et al.*, 2014; Umeki *et al.*, 2013; Yamazaki *et al.*, 2008]

Table 2. Primer sequences used for canine XPO1 and RPL32 genes.

Target gene	Primer name	Sequence (5′–3′)	Tm (°C)	GC %	Product length (bp)
RPL32	RPL32F	TGGTTACAGGAGCAACAAGAAA	63.4	40.9	102
	RPL32R	GCACATCAGCAGCACTTCA	64.1	52.6	
XPO1	XPO1F	TGTGACAGGGCTTTTCAGCT	59.8	50	143
	XPO1R	CCTGTCGAAGGGCTGTTTCT	60	55	

Table 3. IC_{50} concentrations for all canine lymphoma cell lines with KPT-335 treatment.

Cell group	Cell lines	KPT-335 IC_{50} (nM ± SD)
B-cell lines	17-71	89.8 ± 2.07
	CLBL-1	108.6 ± 30.54
	GL-1	294.3 ± 25.57
T-cell lines	CLC	224.7 ± 78.38
	CLGL-90	215 ± 50.96
	Ema	147.8 ± 49.69
	Nody-1	220.5 ± 27.34
	UL-1	418 ± 78.6
Normal	PBMC	872.5 ± 66.72

Table 4. Antibodies for the detection of canine XPO1, p16, p53 and β-actin proteins

Target Protein	Clone	Manufacture	Antibody Dilution Buffer	Dilution
XPO1	D6V7N	Cell Signaling Technology	5% skimmed milk-TBST	1:4000
p16	F-8	Santa Cruz Biotechnology	5% skimmed milk-TBST	1:500
p53	80/p53	BD Transduction Laboratories	5% skimmed milk-TBST	1:1000
β-actin	AC-15	Sigma-Aldrich	5% skimmed milk-TBST	1:5000

Table 5. Combination index (CI) values of canine lymphoma cells treated with combination of KPT-335 and chemotherapeutic drugs (doxorubicin and vincristine) for 24-hour incubation

Cell lines	KPT-335 (nM)	Doxorubicin (nM)	Vincristine (nM)	KPT-335+Dox		KPT-335+Vin	
				Effect (Fa)	CI value	Effect (Fa)	CI value
CLBL-1	62.5	5	0.5	0.541	1.318	0.332	0.207
	62.5	10	1	0.232	0.390	0.272	0.223
	125	5	0.5	0.29	0.626	0.199	0.182
	125	10	1	0.122	0.230	0.195	0.215
GL-1	150	30	1	0.709	0.428	0.392	0.105
	150	60	2	0.631	0.795	0.129	0.378
	300	30	1	0.412	0.281	0.245	0.087
	300	60	2	0.312	0.304	0.114	0.095
Nody-1	125	15	2.5	0.822	0.687	0.461	1.147
	125	30	5	0.789	1.095	0.353	0.728
	250	15	2.5	0.469	0.334	0.175	0.343
	250	30	5	0.435	0.397	0.131	0.242
UL-1	250	125	2.5	0.538	0.456	0.492	0.363
	250	250	5	0.162	0.218	0.398	0.384
	500	125	2.5	0.496	0.525	0.392	0.315
	500	250	5	0.176	0.263	0.356	0.387

FIGURES

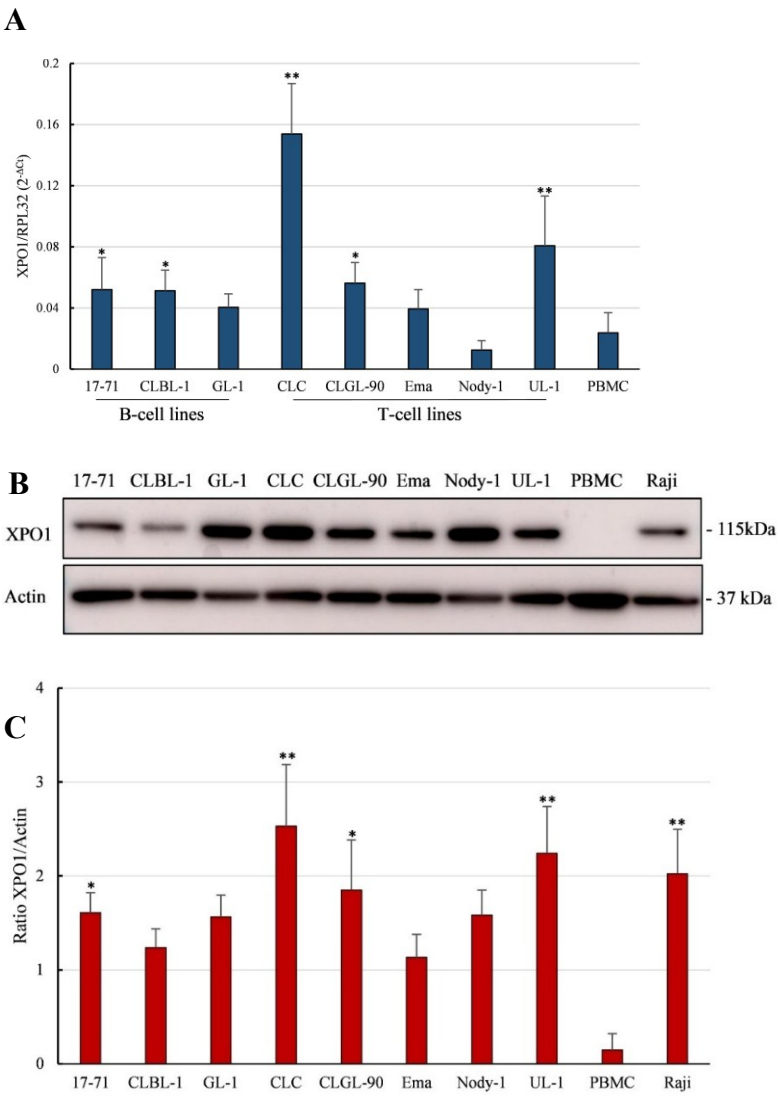

Figure 1. The Exportin 1 (XPO1) gene and protein expressions in canine lymphoma cell lines. A quantitative real-time PCR was performed on eight canine lymphoma cell lines. (A) The Ct values of the XPO1 gene are expressed relative to the ribosomal protein L32 (RPL32) gene. The XPO1 protein expression was assessed using the western blot analysis (B) and quantified using ImageJ (C). Protein expression of β-actin was used as an endogenous control. The data in the panel were presented as mean ± standard deviation (SD) from three independent experiments. *P*-values represented the significant differences of XPO1 gene and protein expressions in canine lymphoma cell lines compared to those of PBMCs obtained from healthy dogs. * $p < 0.05$; ** $p < 0.01$.

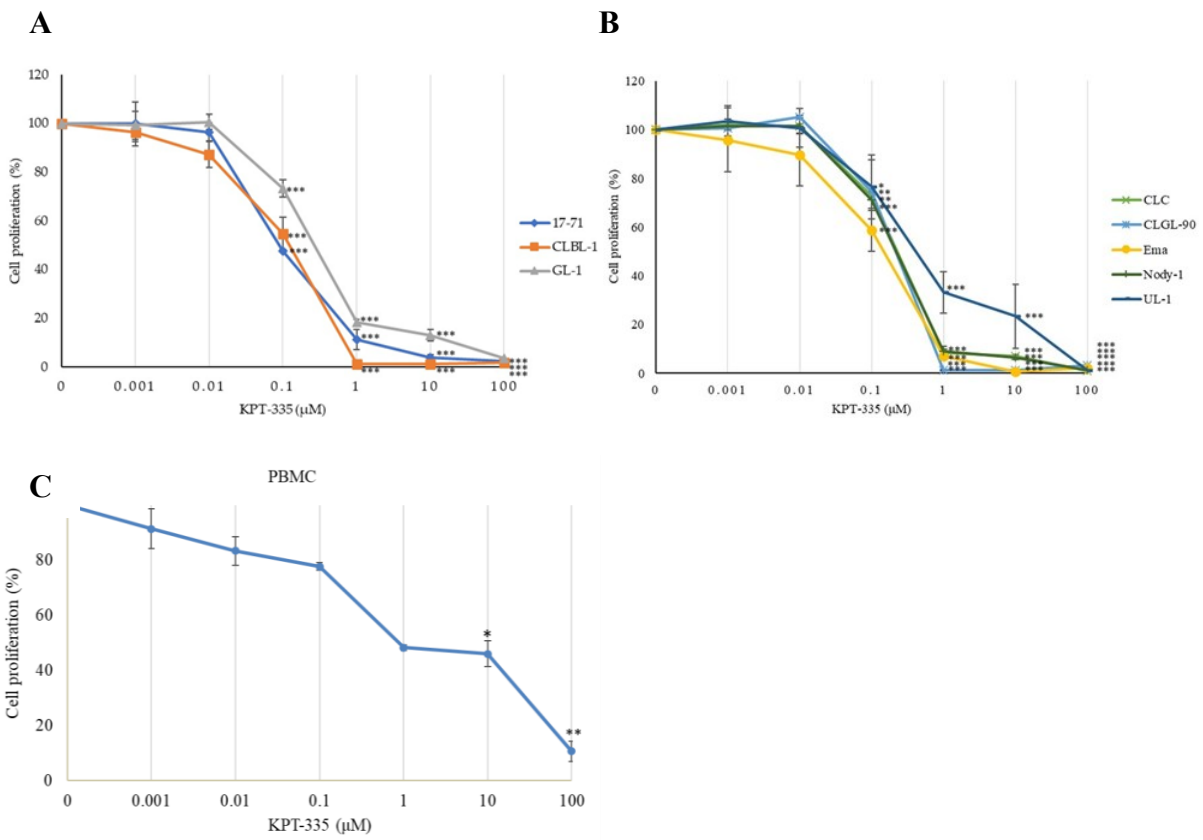

Figure 2. Inhibition of cell proliferation by KPT-335. Canine lymphoma cell lines, B-cell lines (A), T-cell lines (B), and PBMCs (C) were treated with increasing concentrations of KPT-335 for 48 hours. Relative proliferation rates are shown as a percentage of the value of 0 μM as a control. The experiments were performed in triplicate and repeated three times. Error bars indicate SD. *P*-values indicated statistical differences between untreated and treated cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

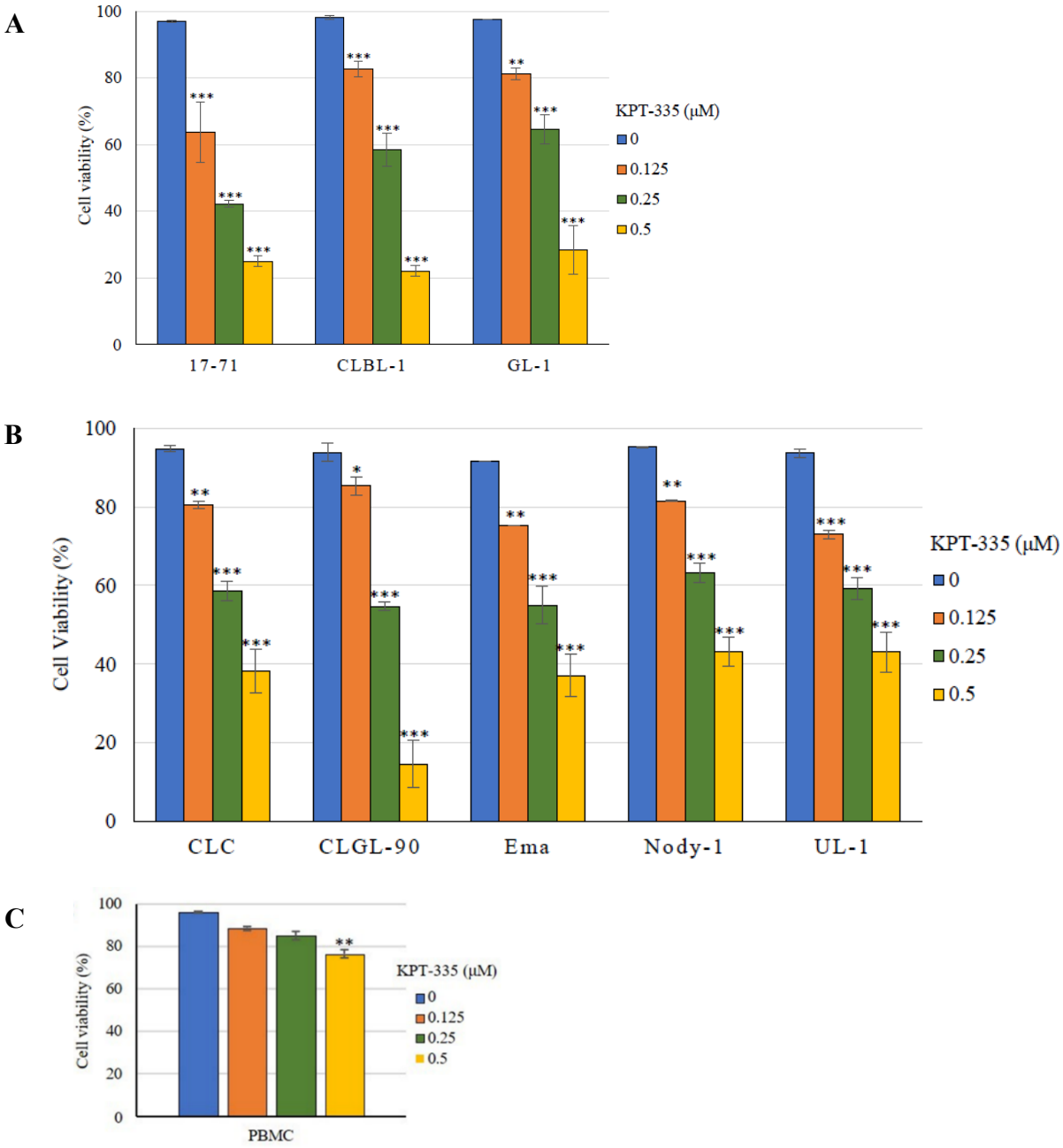

Figure 3. Decreased cell viability by KPT-335. KPT-335 was administered to canine lymphoma cell lines in (A) B-cell lines, (B) T-cell lines, and (C) PBMCs with escalating concentrations (0, 0.125, 0.25 and 0.5 μM) for 48 hours. The cell viability was determined by the percentage of living cells with the trypan blue exclusion method and compared between cells treated with and without KPT-335. The graphs are averages of triplicate wells from three independent experiments, with error bars representing SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

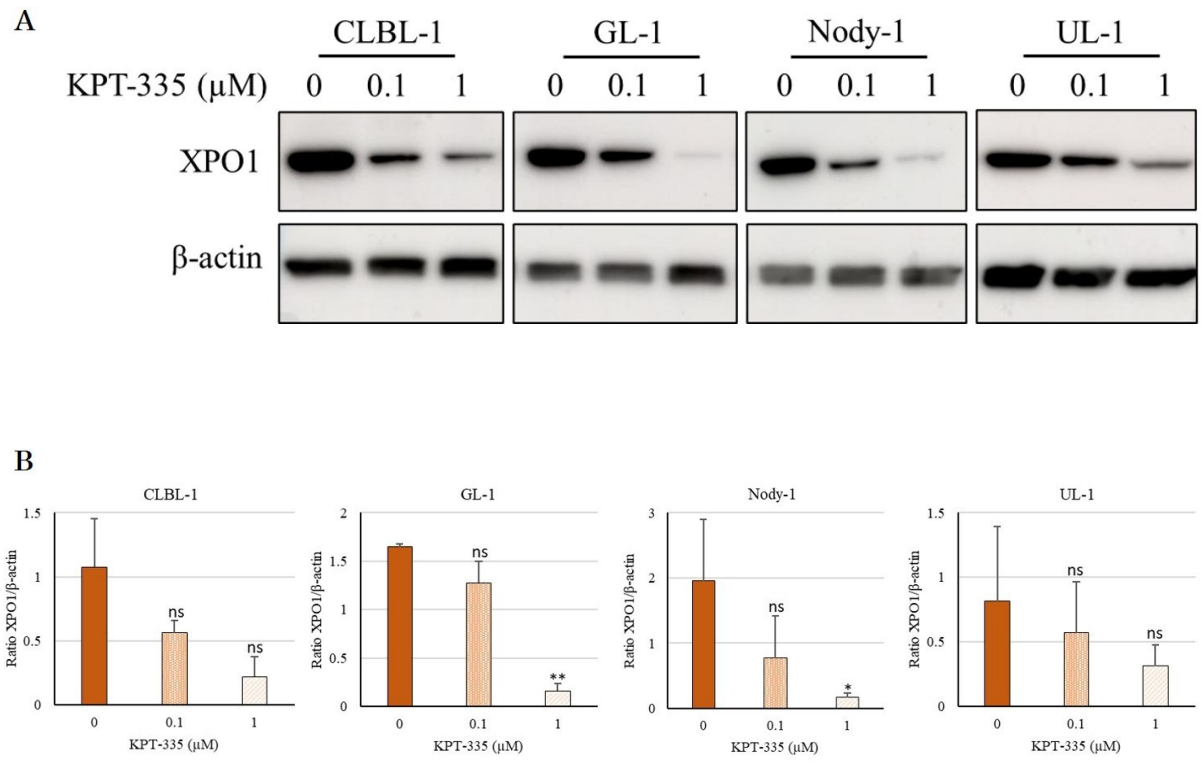

Figure 4. Effect of KPT-335 on Exportin 1 (XPO1) protein expression in canine lymphoma cell lines. Four canine lymphoma cell lines (CLBL-1, GL-1, Nody-1 and UL-1) were treated with DMSO (control), 0.1 μM KPT-335, 1 μM KPT-335 for 24 hours, followed by Western blot analysis of XPO1 protein expression. (A) Representative blots showing dose-dependent reductions in XPO1 expression across all cell lines. (B) Quantification of XPO1/β-actin ratio using ImageJ software. Data were analyzed by one-way ANOVA with Dunnet's *post hoc* test. *$p < 0.05$, **$p < 0.01$, ns: not significant.

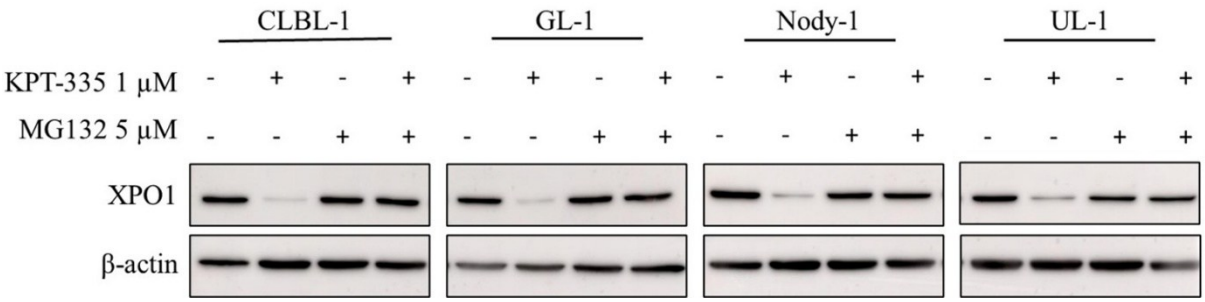

Figure 5. Effect of proteasome inhibition on KPT-335–induced Exportin 1 (XPO1) protein degradation in canine lymphoma cells. Four canine lymphoma cell lines were treated for 6 hours with DMSO (control), 1 μM of KPT-335, 5 μM of MG132, or their combination. Western blot analysis of whole cell lysates showed that MG132 blocked the reduction of XPO1 protein induced by KPT-335, suggesting proteosome-dependent degradation.

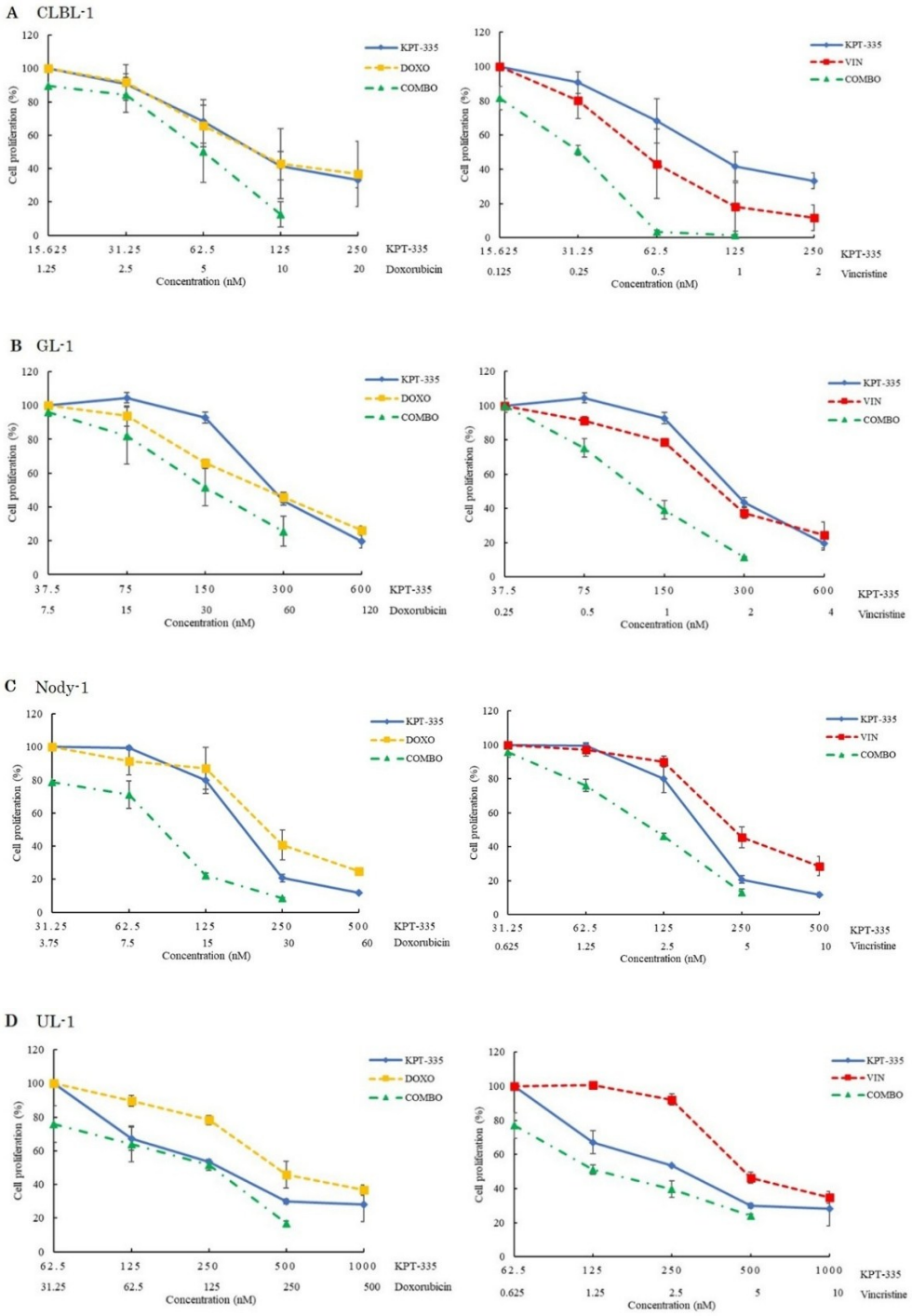

Figure 6. Effect of KPT-335 in combination with doxorubicin or vincristine on cell proliferation in canine lymphoma cell lines. CLBL-1 (A), GL-1 (B), Nody-1 (C) and UL-1 (D) cells were treated for 48 hours with KPT-335, doxorubicin, or vincristine concentrations as single agents, or in combination at approximate IC_{50} and half-IC_{50} as doses. Cell proliferation was quantified and expressed as percentage relative to controls. Data represent mean values from triplicate experiments, repeated independently at least twice.

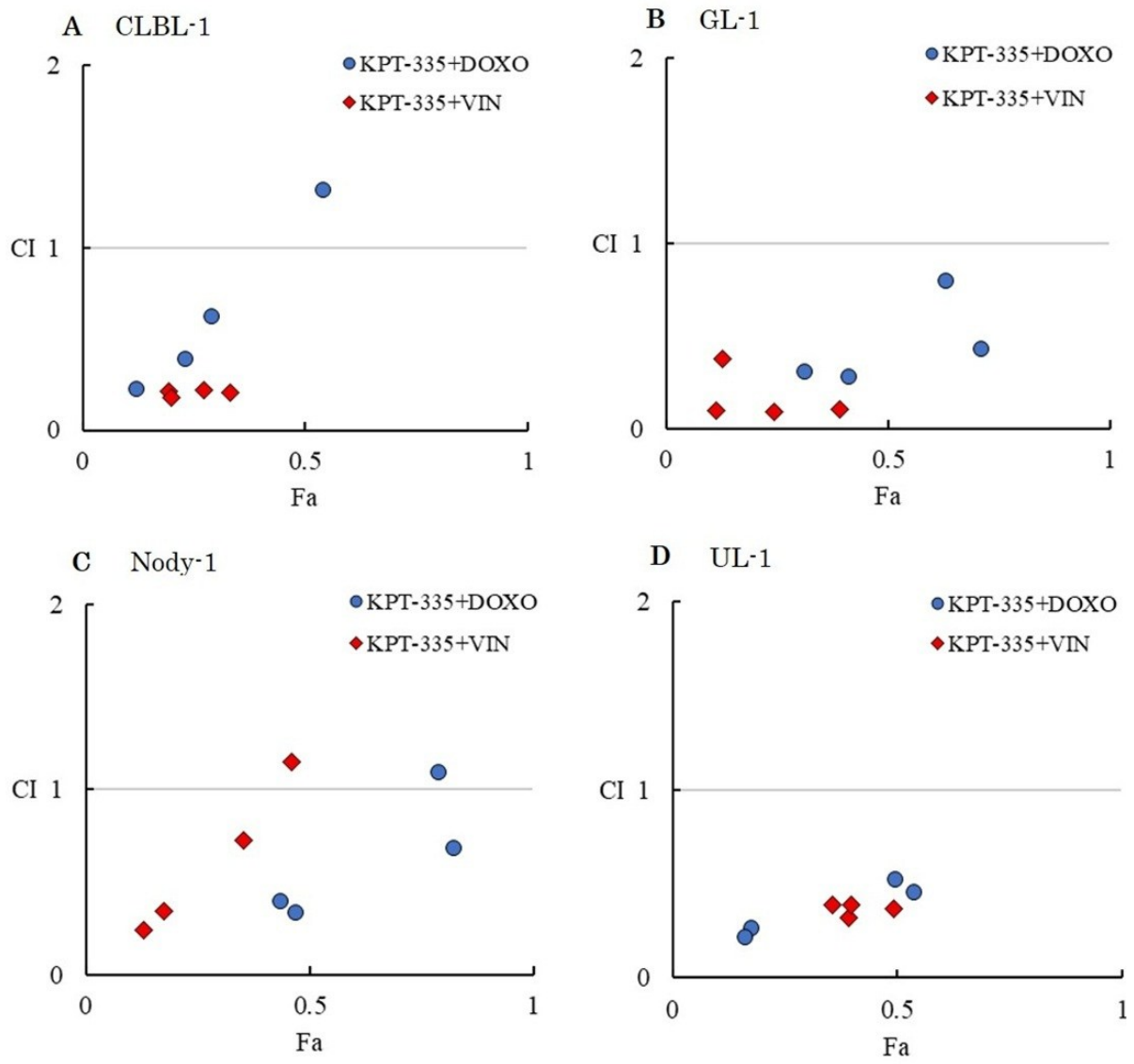

Figure 7. Combination index (CI) plots for KPT-335 in combination with doxorubicin or vincristine in canine lymphoma cell lines. CLBL-1 (A), GL-1 (B), Nody-1 (C) and UL-1 (D) cells were treated for 48 hours with KPT-335, doxorubicin, or vincristine as single agents or in combination. CI values calculated with CompuSyn software, were plotted against the fraction affected (Fa), where Fa = 1 indicates complete growth inhibition, Fa = 0.5 indicates 50% inhibition, and Fa = 0 indicates no effect. CI < 1 denotes synergy, CI = 1 additivity and CI > 1 antagonism. Data represent mean values from triplicate experiments, repeated independently at least twice.

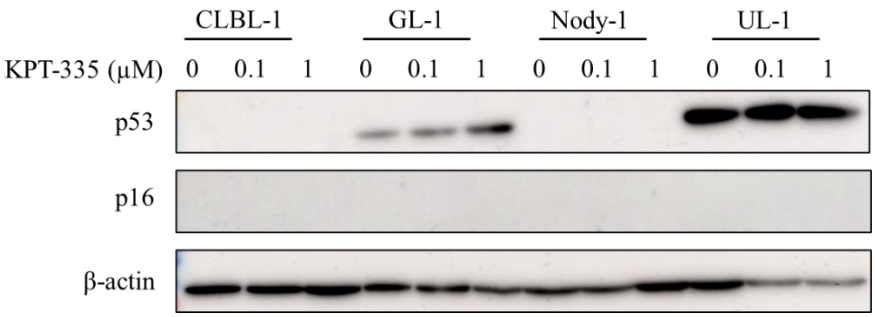

Figure 8. Evaluation of p53 and p16 expression in canine lymphoma cell lines after KPT-335 treatment. Canine lymphoma cell lines were treated with DMSO, 0.1 or 1 μM KPT-335 for 24 hours. Protein lysates were prepared, separated by SDS-PAGE, and analyzed by Western blotting for p53, p16 and β-actin.