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Sensitivity of canine hematological cancers to BH3 mimetics

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Abstract

Background: Inhibition of antiapoptotic B-cell lymphoma 2 (BCL2) proteins by small molecule Bcl-2 homology 3 (BH3) mimetics causes rapid induction of apoptosis of human hematological cancers in vitro and in vivo.

Objectives: Assess in vitro sensitivity of non-neoplastic lymphocytes and primary hematological cancer cells from dogs to venetoclax (VEN) or the dual BCL2/ B-cell lymphoma-extra-large (BCLxL) inhibitor, navitoclax (NAV), and evaluate the association between BCL2 protein expression and VEN sensitivity.

Animals: Nine client-owned dogs without cancer and 18 client-owned dogs with hematological cancer.

Methods: Prospective, nonrandomized noncontrolled study. Lymphocytes isolated from peripheral blood, lymph node, or bone marrow from dogs were incubated with BH3 mimetics for 24 hours. Viable cells were counted using flow cytometry and half maximal effective concentration (EC_{50}) was calculated. BCL2 protein from whole cell lysates was assessed via immunoblots.

Results: Nodal B and T lymphocytes were more sensitive to VEN than circulating lymphocytes ($P = .02$). Neoplastic T lymphocytes were sensitive to VEN (mean $EC_{50} \pm SD = 0.023 \pm 0.018$ μM), whereas most non-indolent B cell cancers were resistant to killing by VEN (mean $EC_{50} \pm SD = 288 \pm 700$ μM). Unclassified leukemias showed variable sensitivity to VEN (mean $EC_{50} \pm SD = 0.49 \pm 0.66$ μM). Detection of BCL2 protein was not associated with VEN sensitivity.

Conclusion and Clinical Importance: Neoplastic canine T lymphocytes are sensitive to VEN in vitro. Quantification of BCL2 protein alone is insufficient to predict sensitivity to VEN.

KEYWORDS
apoptosis, BCL2, lymphoma, small molecule inhibitor

Abbreviations: α-MEM, alpha-minimum essential media; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ANOVA, analysis of variance; BCL2, B-cell leukemia/lymphoma-2; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; EC_{50}, half-maximal effective concentration; EDTA, ethylenediaminetetraacetic acid; FC, flow cytometry; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; ICC, immunocytochemistry; IHC, immunohistochemistry; LN, lymph node; MZL, marginal zone lymphoma; MDCK, Madin-Darby canine kidney; MM, multiple myeloma; NAV, navitoclax; NHL, non-Hodgkin lymphoma; PB, peripheral blood; PBS, phosphate buffered saline; TZL, T-zone lymphoma; VEN, venteoclax

1 | INTRODUCTION

Hematological cancers represent more than 25% of all cancers in dogs, and the disease will relapse in the majority of treated dogs, despite high remission rates after traditional chemotherapy.[1] A commonly identified mechanism associated with resistance to chemotherapy-induced cell death in cancer is the alteration of normal cell death (apoptotic) pathways. The B-cell leukemia/lymphoma-2 (BCL2) family proteins are an important group of regulators that interact to promote or inhibit apoptosis. The antiapoptotic regulators include BCL2, BCLxL, and MCL1.[2] These proteins prevent cell death by binding and inhibiting the proapoptotic effector proteins BAX and BAK.[3] Regulating the effects of the antiapoptotic BCL2 proteins are the BH3-only proteins, which bind to and inhibit BCL2 proteins, enabling damaged or stressed cells to undergo apoptosis.[3] Overexpression of BCL2 occurs in numerous hematological cancers in people including chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), non-Hodgkin lymphoma (NHL), and multiple myeloma (MM).[4-8]

Evaluation of antiapoptotic proteins in dogs with lymphoma is limited, but existing reports suggest BCL2 overexpression in some disease subsets. One study demonstrated almost universal expression of BCL2 via immunohistochemistry (IHC) of lymph node (LN) biopsies in dogs with diffuse large B-cell lymphoma (DLBCL).[9] Another group utilized flow cytometry of lymph node aspirates taken from dogs with T and B cell lymphoma and found the BCL2:BAX ratio to be significantly higher in malignant T-cells compared with malignant B cells and to lymphocytes from healthy dogs.[10] BH3 mimetics are small molecule inhibitors of BCL2 family proteins, and include the dual BCL2 and BCLxL inhibitor navitoclax (NAV) and the BCL2-specific inhibitor venetoclax (VEN), which was developed after the observation of dose-limiting thrombocytopenia in both people and dogs treated with NAV, secondary to the reliance of platelets on BCLxL for survival.[11] Venetoclax has activity against CLL, AML, NHL, and MM in people, and is approved for treatment of CLL and AML in the United States and Australia.[5,12] Utilization of ex vivo drug sensitivity screening with BH3 mimetics can predict clinical responses in vivo.[13-17] These studies report a strong association between half-maximal effective concentration (EC_{50}) and clinical response, whereby an EC_{50} <100 nM is readily achievable in vivo and results in effective cell killing, whereas >1000 nM indicates cells are relatively resistant to killing and the agent is unlikely to be associated with single-agent efficacy in vivo.[11,16]

We aimed to utilize functional viability assays to characterize the in vitro sensitivity of normal canine lymphocytes, as well as primary canine hematological cancers, to BH3 mimetics. We further aimed to evaluate expression of BCL2 in these diseases and to assess the association between BCL2 protein expression and VEN sensitivity. We hypothesized that some canine hematological cancer cells would be sensitive to VEN, and that this would be associated with expression of BCL2.

2 | MATERIALS AND METHODS

2.1 | Cell lines and culture conditions

The human B-cell acute lymphoblastic leukemia cell line RS4;11 and human plasmacytoma cell line AMO-1 were obtained from the ATCC and Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) respectively, and were cultured in Roswell Park Memorial Institute (RPMI, ThermoFisher, Massachusetts) media plus 10% FCS, 100 U/mL penicillin, and 10 mg/mL streptomycin. Their identity was genetically confirmed before use. A *BCL2* knock-out AMO-1 cell line had been generated previously using CRISPR/Cas9 technology and validated as described.[18] The canine osteosarcoma cell line OSCA40 (donated by Dr Christine Hawkins) was maintained in alpha-minimum essential media (α-MEM, ThermoFisher, Massachusetts) with 10% fetal calf serum (FCS, ThermoFisher, Massachusetts), supplemented with 10 mM L-glutamine, 100 U/mL penicillin and 10 mg/mL streptomycin. Madin-Darby canine kidney (MDCK) immortalized canine kidney cells were originally obtained from the American Type Culture Collection (ATCC, Virginia) and were maintained in Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher, Massachusetts) plus 10% FCS, 100 U/mL penicillin, and 10 mg/mL streptomycin. All cell lines were cultured at 37°C with 5% CO_2 and were limited to four passages before cells were discarded. Cell lines were subject to regular testing for *Mycoplasma* contamination (Mycoalert Mycoplasma Detection Assay, Lonza, Maryland) and returned negative results in all instances.

2.2 | Enrolment and sample processing

Dogs with a cytologic diagnosis of multicentric lymphoma, MM, or leukemia were eligible for inclusion. Dogs without a known history or diagnosis of cancer were included for assessment of non-neoplastic lymphocytes. Owners of dogs participating in the study provided informed consent for enrolment. The study was approved by The University of Melbourne Animal Ethics Committee. A summary of the diagnostic criteria for included cases can be found in the Appendix S1.

Peripheral LN aspirates and peripheral blood (PB) were collected from 9 dogs without cancer to assess sensitivity of normal lymphocytes to VEN and NAV. For the cell viability assay, peripheral LN aspirates were obtained using a 22-gauge needle collected into sterile tubes containing Iscove's Modified Dulbecco's Medium (IMDM, ThermoFisher, Massachusetts) cell culture medium +10% FCS and were stored at 4°C for up to 24 hours before cryopreservation or analysis. Five milliliters of venous blood was collected into sterile ethylenediaminetetraacetic acid (EDTA)-containing tubes and stored at room temperature for up to 24 hours before mononuclear cell isolation. Bone marrow aspirates were also collected into sterile EDTA tubes and stored and handled in the same way as peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich, UK), as

previously described.[19] Lymph node pellets were washed using a Red Cell Removal Buffer (156 mM NH_4Cl, 11.9 mM $NaHCO_3$, 0.097 mM EDTA, Walter and Eliza Hall Institute Media Kitchen, Australia) to remove red blood cells. Cells were either utilized fresh (n = 14), or stored at −80°C in IMDM +20% FCS + 20% dimethyl sulfoxide (DMSO) as previously described for human primary mononuclear cells[20] and thawed before analysis (n = 13). Cells were thawed by placing frozen vials in a 37°C water bath and agitated for 1 to 2 minutes. Cells were slowly resuspended in chilled thawing media, composed of IMDM +20% FCS + 20% DMSO. All samples were washed and resuspended in warmed complete media, and viability was assessed by using trypan blue (0.2%) exclusion at a 1:1 ratio, and counted using an automated hemocytometer (TC20, BioRad, New South Wales, Australia). Samples with a baseline viability of <50% were excluded from the analysis.

2.3 | Cryopreservation assessment

Use of frozen mononuclear cells for ex vivo drug sensitivity testing has been previously evaluated in human samples and no effect on drug sensitivity has been reported.[16] However, as this has not been assessed in canine samples, we collected LN aspirates from 4 dogs with multicentric lymphoma and performed the drug sensitivity assay on fresh cells, froze the remaining cells as described, and repeated the assay after thawing to compare the EC_{50} between fresh and thawed samples.

2.4 | In vitro BH3 mimetics sensitivity cell kill assay

Figure S1 summarizes the sample processing and experimental pathway. Primary samples from dogs with cancer were seeded into a 96-well plate at 1×10^5 cells/well in triplicate, whereas primary samples from dogs without cancer were seeded at 6.5×10^4 to 1×10^5 cells/well in triplicate, depending on cellular yield. Because of limited cell numbers, the assay was performed once for each primary sample. The RS4;11 cell line, which has consistent sensitivity to BH3 mimetics, was used as a positive control with each experiment.[11,21] Cells were seeded at 1×10^5 cells/well in single replicates. Either VEN or NAV (AbbVie, North Chicago, Illinois), dissolved in DMSO to a final concentration of 1 mM, were added to cells at graded drug concentrations (2 nM to 10 μM). Negative control wells were included using DMSO at 0.001%. Plates were incubated for 24 hours at 37°C at 5% CO, based on the maximal time point of cell killing of human CLL cells.[21] Primary cells were counted by flow cytometry on a CytoFLEX (Beckman Coulter, Indianapolis, Indiana) analyzer. Selection of neoplastic cells was based on identification of a homogenous cell population with similar forward scatter properties, and surface immunophenotyping with anti-CD3 fluorescein isothiocyanate (FITC, clone CA17.2A12, Bio-Rad Laboratories, NSW, Australia) and anti-CD21 AlexaFluor647 (AF647, clone CA2.1D6, Bio-Rad Laboratories,

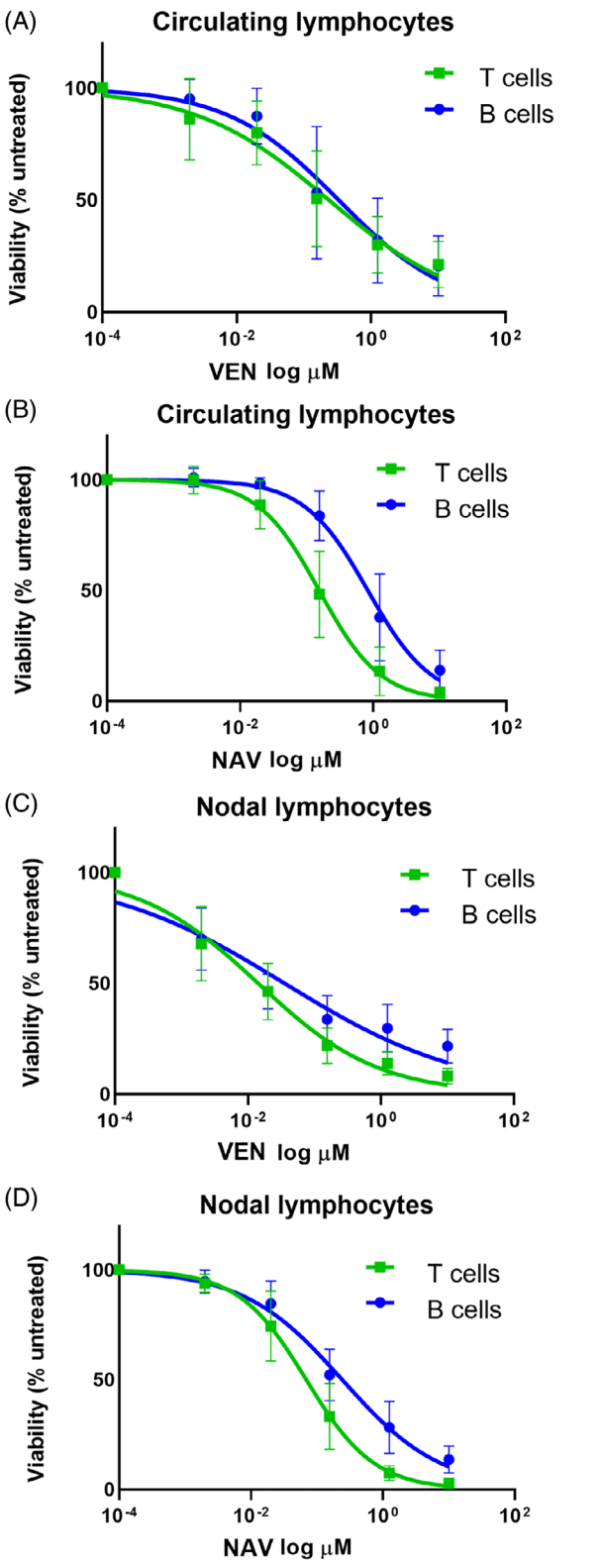

FIGURE 1 Summary cell viability data for normal canine CD3+ T and CD21+ B lymphocytes after 24 hours treatment with VEN and NAV, with viability normalized to DMSO control wells. Points and error bars represent the mean EC_{50} ± SD. Viability of peripheral blood (A and B) from 8 dogs, and lymph node (C and D) aspirates from 5 dogs is shown

TABLE 1 Estimated mean EC_{50} ± SD for normal lymphocytes isolated from peripheral blood (8 dogs), and lymph node (5 dogs), after 24 hours treatment with VEN and NAV, calculated from dose–response curves presented in Figure 1

	Estimated mean EC_{50} ± SD (nM)			
Site	B cells VEN	T cells VEN	B cells NAV	T cells NAV
Peripheral blood (n = 8)	1140 ± 1550	525 ± 650	1220 ± 1080	220 ± 190
Lymph node (n = 5)	92 ± 140	31 ± 24	430 ± 440	112 ± 97

NSW, Australia) antibodies. Propidium iodide (PI) exclusion was used to identify viable cells, with a minimum of 10 000 events per sample. Viability was reported as a percentage of viable cells in the associated DMSO negative control wells. Flow cytometry data was analyzed using Flow Jo version 10 software (TreeStar Inc., Ashland, Oregon). Figure S2A,B illustrate the gating strategy used for primary cells and Figure S2C-E summarizes the control data using RS4;11, MDCK, and OSCA40 cell lines.

2.5 | Immunoblot assessment of BCL2

Immunoblot of BCL2 was performed as described[22] and a detailed description of the methodology can be found in the Appendix S1.

2.6 | Statistical analysis

EC_{50} values were derived by fitting dose-response curves of normalized cell viability against log concentration of BH3 mimetic using a sigmoidal dose-response equation, as previously described.[13] Data normality was assessed using the Shapiro-Wilk test and was normally distributed for all groups except B cell cancers treated with NAV. Comparison of two normally distributed groups was performed using a two-sided Student's *t*-test, with a *P*-value at <.05. One-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons was performed when comparing three or more groups. All statistical analyses were performed using Graphpad Prism version 9.0 (Graphpad Software, Inc., San Diego, California).

3 | RESULTS

3.1 | Sensitivity of normal canine B and T lymphocytes to BH3 mimetics

Peripheral blood was analyzed from 8/9 dogs (1 sample was poorly cellular and results were excluded), while lymph node aspirates were collected and analyzed from 5/9 dogs. Animal characteristics and lymphocyte sensitivity to VEN and NAV are summarized in Table S1. Seven of the PB and 4 of the LN samples had sufficient cells for each assay to include three replicates.[13] Cell numbers were lower in 1 PB and 1 LN sample, so only single assessments were performed at each drug concentration. Summary dose response curves showing mean EC_{50} ± SD are presented in Figure 1A-D and described in Table 1. Individual dog sensitivity data is shown in Figure S3A-H. Nodal B cells were more sensitive to VEN than circulating B cells (Figure 2A), but this observation was not seen for NAV (Figure 2C). Similarly, lymph node-derived T cells were more sensitive to VEN than circulating T cells (Figure 2B) but no difference in sensitivity was seen for NAV for T cells (Figure 2D). Paired comparisons showed that nodal B and T cells were more sensitive to VEN than NAV (Figure 3A,B); however, differential BH3 mimetic sensitivity was not observed for circulating B or T cells (Figure 3C,D).

3.2 | Characteristics of dogs with hematological cancers

From April 2019 to October 2021 samples were collected from 30 dogs with lymphoma, leukemia, or myeloma. Of these, 18 dogs were included in the final analysis. Reasons for the exclusion from final analysis for 12 dogs are summarized in Table S3. Of the dogs included in the final analysis, there were 6 dogs with multicentric intermediate to large B-cell lymphoma, 3 dogs with multicentric intermediate to large T-cell lymphoma, 4 dogs with unclassified leukemia, and 1 dog with MM. Signalment, disease, method of diagnosis, and viability assay details are summarized in Table S2.

To assess whether the technical step of thawing DMSO-cryopreserved primary cells altered BH3 mimetic sensitivity, the viability assay was performed using lymph node aspirates from 4 dogs with multicentric lymphoma, comparing paired fresh and thawed samples. No difference between mean EC_{50} for either VEN or NAV was observed, and dose-response curves at clinically relevant concentrations appeared similar (Table S4, Figure 4). Given the previously reported data in primary human cells indicating careful thawing did not alter their sensitivity,[20] along with our findings, we concluded use using either cryopreserved or fresh primary cells for functional viability assays is a valid approach.

3.3 | Canine B-cells and cancers show resistance to BH3 mimetics

We found that 6/7 (86%) B cell cancers showed resistance to treatment with BH3 mimetics (Figure 5, Table S2) with a mean estimated EC_{50} ± SD for VEN 288 ± 700 μM and NAV 225 ± 540 μM. Malignant B cells were more resistant to VEN than normal nodal B cells ($P = .003$), but not normal circulating B cells ($P = .6$, Figure 6). Malignant B cells did not differ in sensitivity to NAV compared with

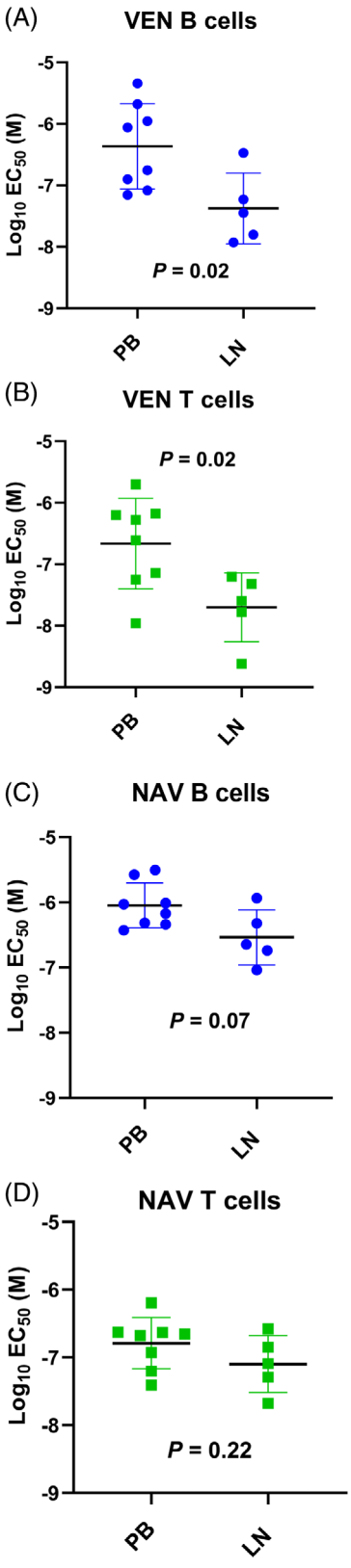

FIGURE 2 In vitro sensitivity of peripheral blood (PB) and lymph node (LN) derived B lymphocytes (A) and T lymphocytes (B) from individual dogs to VEN and for the same cells to NAV (C and D). Data show the log-transformed EC_{50}, with the horizontal line representing the mean and error bars showing SD. *P*-values derived from unpaired Student's *t*-test

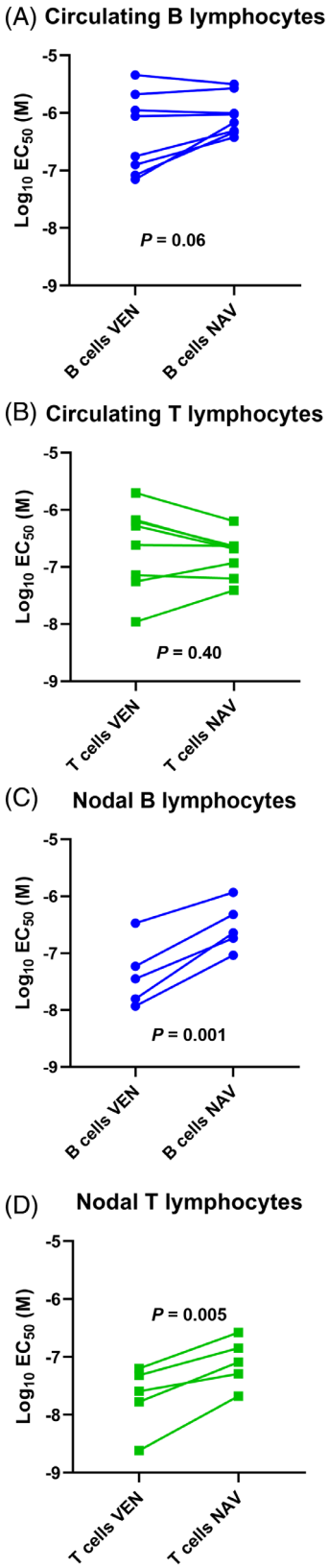

FIGURE 3 Paired comparisons of normal lymphocyte sensitivity to VEN and NAV. Connecting lines show sensitivity of lymphocytes from an individual dog treated under the same conditions, comparing different BH3 mimetics. Data represent log-transformed EC_{50} from individual dogs, with the horizontal line representing the mean and error bars showing SD. *P*-values derived from paired Student's *t*-test

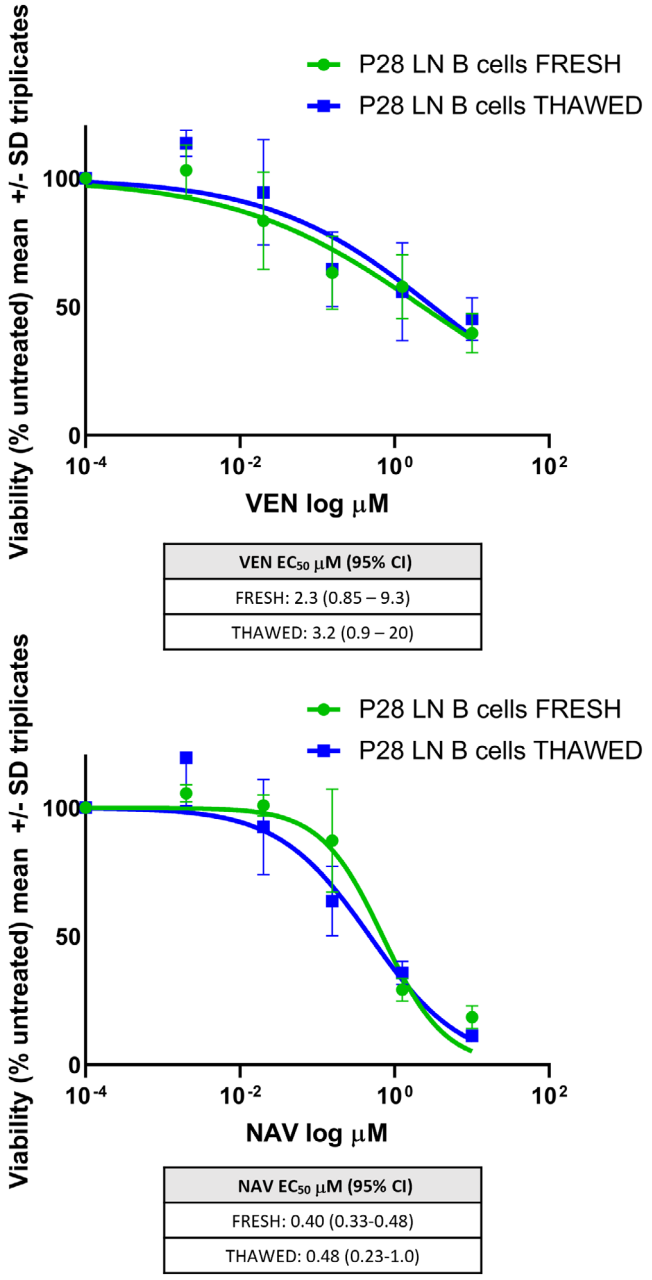

FIGURE 4 Representative dose response curve from 1 dog (dog 28) showing CD21+ B lymphocytes after 24 hours incubation with VEN or NAV. Data shown represent mean ± SD from triplicate wells and are normalized to the DMSO control

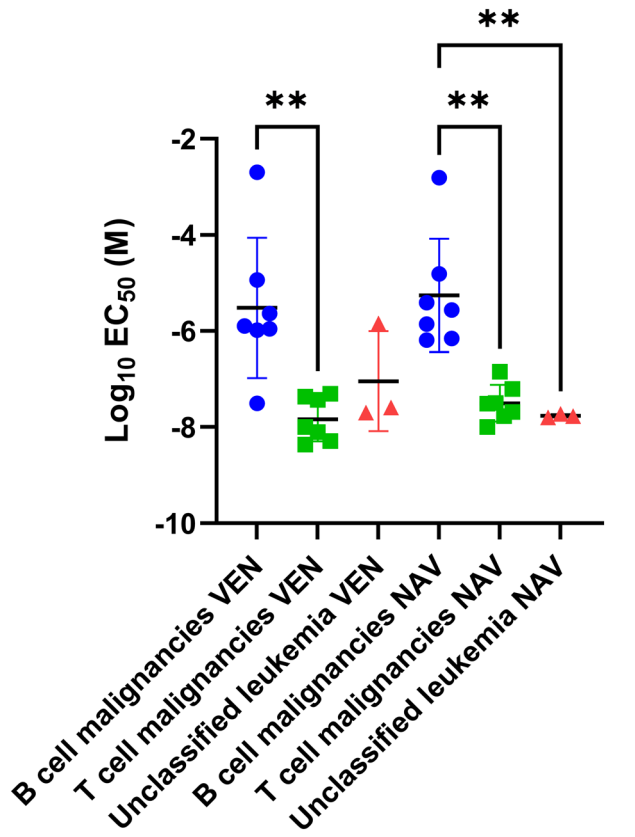

FIGURE 5 Dot plot comparing the in vitro sensitivity of canine B and T cell cancers and unclassified leukemia to BH3 mimetics. Data represent the log transformed EC_{50} with the mean (horizontal line) and SD (error bars) after 24 hours incubation with VEN or NAV. Groups were compared using 1-way ANOVA. ** Indicates a *P*-value <.01

normal nodal B cells ($P = .17$) or normal circulating B cells ($P = .71$). Figure 7A shows the lymphocyte viability curve from 1 sample (dog 37) with advanced nodal MZL without peripheral blood disease involvement and illustrates the sensitivity of normal circulating CD3+ T cells and CD21+ B cells compared with neoplastic CD21+ nodal cells. The majority of evaluated aggressive B cell cancers were resistant to cell killing by VEN and NAV. The exception was dog 14 that was diagnosed cytologically with multicentric large CD21+ B-cell lymphoma. The nodal lymphocytes from this dog showed sensitivity to VEN (EC_{50} 31.4 nM, Table S2).

3.4 | Canine T-cell cancers show sensitivity to BH3 mimetics

Canine T-cell cancers showed consistent sensitivity to BH3 mimetics, with 3/3 dogs with multicentric intermediate-large T-cell lymphoma, 2/2 T-CLL, and 2/2 TZL showing a mean EC_{50} ± SD for VEN of 23 ± 18 nM, and for NAV of 45 ± 42 nM (Figure 6, Table S2). Differential sensitivity to VEN or NAV was not observed ($P = .05$). T cell cancers were more sensitive to both VEN and NAV compared with B cell cancers ($P = .001$ and $P = .002$, respectively, Figure 5). Figure 7B shows an example cell viability curve for nodal lymphocytes from dog 26 after a diagnosis of multicentric T cell lymphoma, illustrating the marked sensitivity of neoplastic nodal CD3+ T cells to BH3 mimetics. No difference in sensitivity to VEN or NAV was seen between T cell cancers and unclassified leukemias ($P = .83$ and $P > .99$, respectively). Malignant T cells did not differ in VEN sensitivity compared with circulating normal T cells ($P = .13$) or normal nodal T cells ($P > .99$, Figure 6). Similarly, no difference in sensitivity to NAV was seen between malignant T cells and normal circulating T cells ($P = .83$) or nodal T cells ($P > .99$, Figure 6). In summary, canine T cell cancers show consistent sensitivity to VEN.

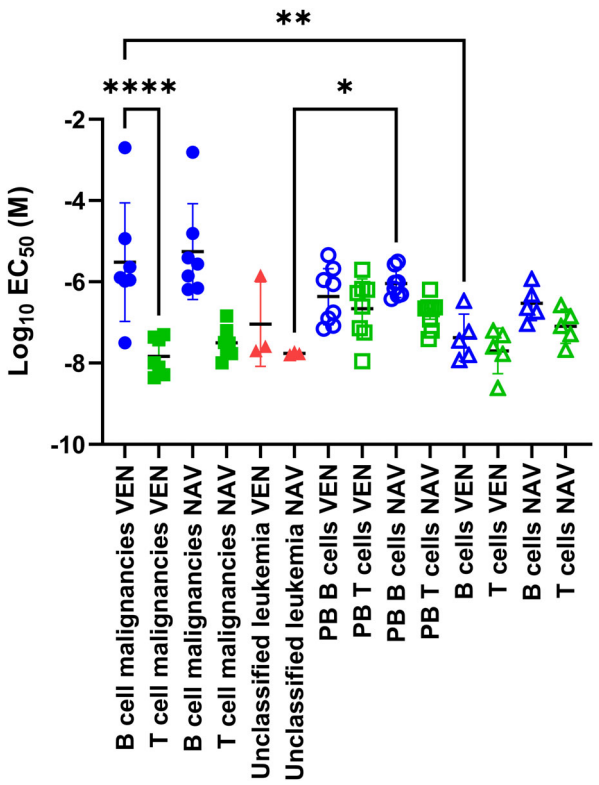

FIGURE 6 Dot plot comparing the in vitro sensitivity of canine B and T cell cancers, unclassified leukemia and normal lymphocytes to BH3 mimetics. Data represent the log transformed EC_{50} with the mean (horizontal line) and SD (error bars) after 24 hours incubation with VEN or NAV. Groups were compared using 1-way ANOVA. * Indicates a *P*-value <.05; ** indicates a *P*-value <.01; **** indicates a *P*-value <.0001

3.5 | Canine unclassified leukemias show sensitivity to navitoclax

Four dogs with unclassified leukemias were included in the final analyses. The mean EC_{50} ± SD for VEN was 490 ± 660 nM, and for NAV was 17 ± 0.12 nM. Comparison of mean EC_{50} between disease subsets revealed that unclassified leukemias were more sensitive to NAV than B cell, but not T cell, lymphoma, and chronic leukemias ($P = .01$, Figure 5). No differences were seen in sensitivity to VEN between unclassified leukemias and B or T cell lymphoma and chronic T cell leukemia (Figure 5). Unclassified leukemias did not differ in VEN or NAV sensitivity compared with normal B or T lymphocytes, except for circulating B cells, which were more resistant to NAV compared with unclassified leukemias ($P = .05$, Figure 6).

3.6 | BCL2 expression and venetoclax sensitivity

To assess whether BCL2 expression was associated with VEN sensitivity, immunoblotting was performed for 10 canine patient samples and 4 cell lines. The VEN-sensitive RS4;11 cell line was used as a positive control, while the *BCL2* knock-out AMO-1 cell line was included as a negative control in each blot. Canine MDCK and OSCA40 cell lines demonstrated resistance to VEN and NAV in vitro (Figure S2D-E) and lysates from these cells were included as controls in 1 blot. Primary samples included 3 T-cell cancers (1 TZL, 1 T-CLL, and 1 T cell lymphoma), 3 B cell lymphoma (including 1 MZL), and 4 unclassified leukemias (including 1 VEN-resistant myeloid leukemia and 1 VEN-sensitive myeloid leukemia). The results of the immunoblotting are

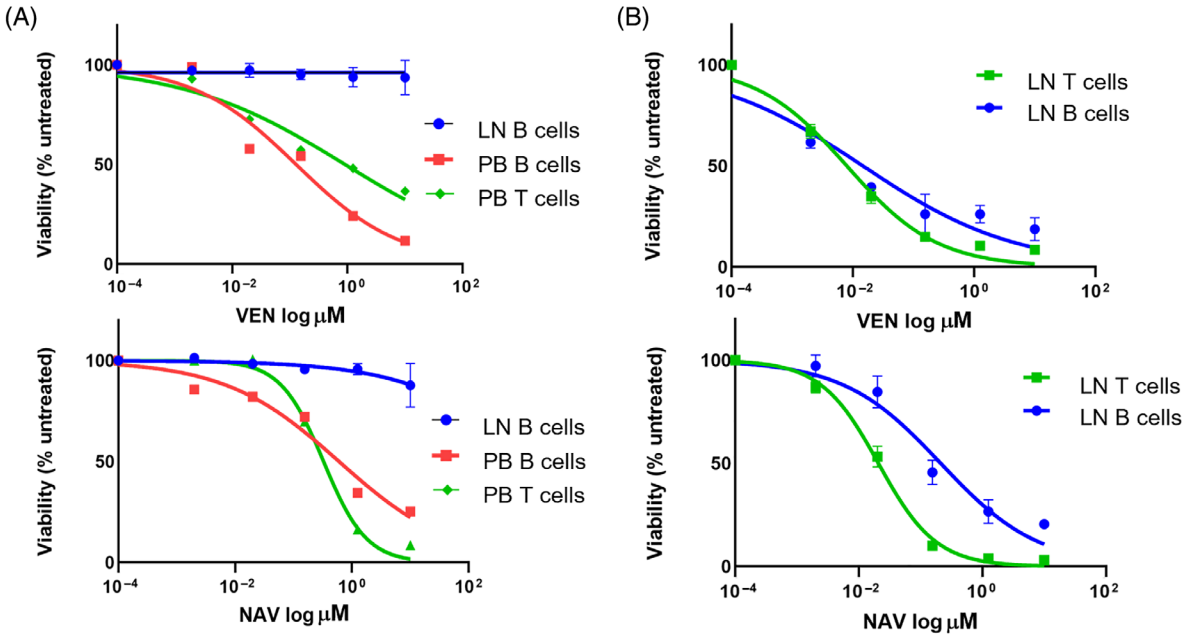

FIGURE 7 Dose response curves to VEN and to NAV showing mean viability of cells normalized to DMSO control ± SD of triplicate wells. (A) Dog (37) with advanced nodal marginal zone lymphoma. The EC_{50} of CD21+ neoplastic nodal cells exceed 5 μM. (B) Dog (26) with multicentric large T cell lymphoma. The EC_{50} of neoplastic CD3+ nodal T cells to VEN and NAV are 7.9 and 20.8 nM, respectively

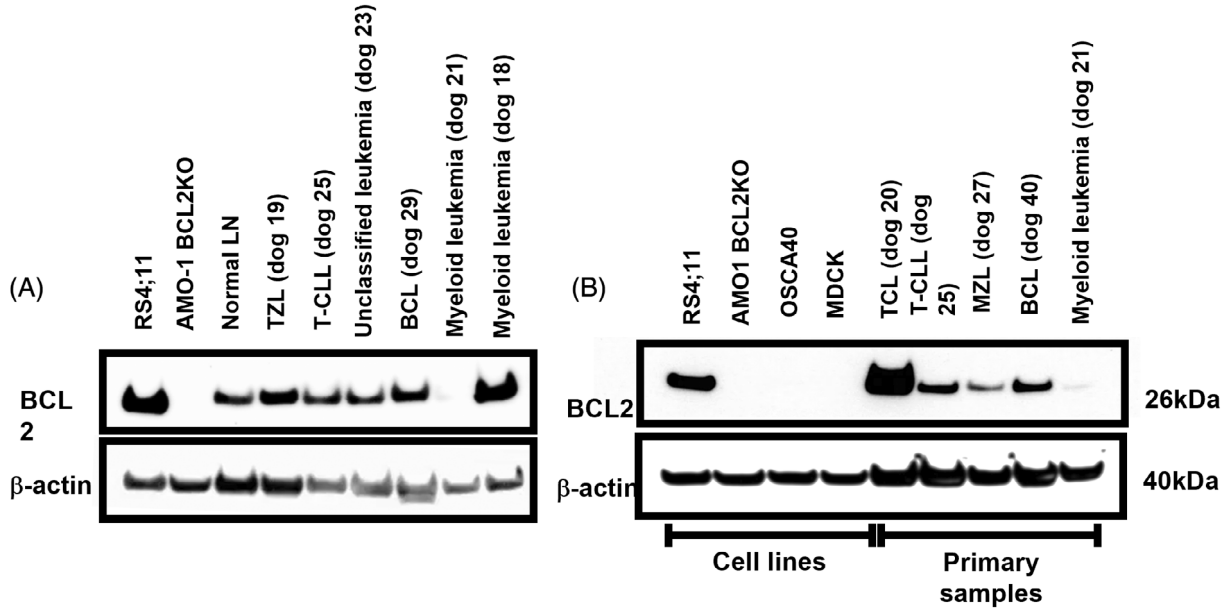

FIGURE 8 Two immunoblots showing canine BCL2 protein expression and β-actin loading control for T and B cell cancers and unclassified leukemias, with sample number in parentheses. Each blot includes positive (RS4;11) and negative (AMO-1 BCL2 knock out) controls for BCL2 expression. (A) Normal lymph node (LN) shows moderate BCL2 expression; all VEN-sensitive samples (dog 19, 20, 25, 23, and 18; EC_{50} < 50 nM) show moderate to strong BCL2 expression. The VEN-resistant B cell lymphoma sample (dog 29; EC_{50} 1.04 μM) shows moderate to strong BCL2 expression, whereas the VEN-resistant myeloid leukemia sample (dog 21; EC_{50} 1.65 μM) shows little to no BCL2 expression. (B) The VEN-resistant solid canine tumor cell lines OSCA40 and MDCK (EC_{50} > 50 μM) do not express detectable BCL2. The primary sample marginal zone lymphoma (dog 27; EC_{50} 1.84 μM) displays low-level BCL2. The VEN-resistant B cell lymphoma sample (dog 40; EC_{50} 1.27 μM) displays moderate level BCL2

shown in Figure 8. The VEN-resistant samples varied in their BCL2 expression—cells from dog 21 (myeloid leukemia) showed little to no BCL2 expression, whereas all B cell lymphoma samples had detectable BCL2 expression, although the degree was variable and lowest in the most resistant sample (dog 27). Cell lines MDCK and OSCA40 had no detectable BCL2 expression. In contrast with the VEN-resistant myeloid leukemia sample, the VEN-sensitive myeloid leukemia sample (dog 18) showed high BCL2 expression. In both VEN-sensitive T cell cancers assessed, stronger BCL2 expression was seen in the T cell lymphoma sample (dog 20) compared with the T-CLL sample (dog 25), despite similar VEN EC_{50} (5.1 and 4.3 nM, respectively). These results were consistent with the hypothesis that BCL2 expression was necessary for cell killing by VEN. They further indicate that presence of detectable BCL2 protein is insufficient for VEN sensitivity.

4 | DISCUSSION

Canine T cell cancers showed consistent and marked sensitivity to BH3 mimetics, with targeting of BCL2 alone with VEN being typically as effective as dual targeting of BCL2 and BCLxL by NAV. To date, VEN treatment of human T cell cancers has not been explored extensively in clinical trials. Expression of BCL2 in human T cell cancers appears to vary amongst disease subsets. For example, positive BCL2 expression as determined via IHC in human peripheral T cell lymphoma not-otherwise-specified (PTCL-NOS) can be seen in 43% to 79.5% of cases, and could be greater in ALK-negative, compared with ALK-positive, anaplastic large cell lymphoma.[23-25] The role of VEN in management of T cell cancers in people is still being defined, though several reports suggest activity in combination with other therapies including the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib, and histone deacetylase inhibitors.[26,27] Current standard of care treatment for aggressive canine T cell lymphoma involves multiagent maximum tolerated dose chemotherapy. With treatment, overall response rates up to 80% to 97% can be seen, however responses are typically short-lived, with the majority of dogs experiencing disease progression by 200 days.[1,28,29] There is an unmet need for novel therapies to improve outcomes for dogs with aggressive T cell cancers. Our data suggest that canine T cell cancers are often sensitive to cell killing with VEN at low nanomolar concentrations. Pharmacokinetic, dose-finding safety, and efficacy trials are therefore warranted to assess these effects in vivo in dogs with spontaneous T cell cancers.

The majority of evaluated B cell cancers from dogs showed resistance to VEN, with 6 of 7 (86%) cases having an EC_{50} >1 μM. This finding is consistent with a recent abstract that reported an IC_{50} of 2.7 μM and 12.3 μM for two canine B cell lymphoma cell lines treated with VEN.[30] We did observe a range of EC_{50} values amongst primary B cell lymphoma samples from 0.03 to >50 μM, which suggests variable BCL2 dependence among canine B cell cancers. Whereas VEN has shown promise in the treatment of B cell cancers in people, the subsets of diseases that have benefited most tend to be indolent B cell cancers, such as B-CLL, follicular lymphoma and mantle-cell

lymphoma.[31,32] Only 2 dogs with B cell lymphoma had a complete histopathological diagnosis. This means we could not assess differential BH3 mimetic sensitivity amongst lymphoma subtypes, as has been done for people. In our study, all cases demonstrated an aggressive clinical behavior, rapid LN enlargement, and progression, and showed response to CHOP chemotherapy. It is therefore unlikely that any indolent cases were included, so the sensitivity of canine indolent lymphoma to BH3 mimetics remains an open question.

The finding that non-indolent canine B cell lymphoma is largely VEN-resistant was not entirely unexpected. Whereas single-agent treatment with VEN in human DLBCL (hDLBCL) produces an overall response rate of only 18%, despite high BCL2 expression on IHC.[32] Further, several hDLBCL cell lines harboring the t(14;18) translocation that results in BCL2 overexpression still show resistance to VEN.[11] However, a recent phase 2 trial reported that combination of VEN with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) as a front-line therapy improved progression-free survival in treatment-naïve hDLBCL when indirectly compared with historical controls that received R-CHOP alone.[33] Particular benefit was seen in people with BCL2-positive MYC-positive disease, so-called double hit lymphoma—a group that historically has benefited less from traditional R-CHOP therapy—however, these results are still to be validated in phase 3 clinical trials.[32,33] Investigation into potential synergistic interactions between VEN and CHOP in B cell lymphoma in dogs is warranted.

It has been hypothesized that hDLBCL may up-regulate other antiapoptotic proteins, such as MCL1, such that it is no longer reliant on BCL2 expression for survival. This is being explored through the development of MCL1 inhibitors, which are currently in early-phase clinical trials. Our findings do not support BCL2-dependence in the majority of non-indolent canine B cell cancers, thus further investigation into the expression of other antiapoptotic proteins, including MCL1, is warranted.

Venetoclax is approved for the treatment of AML in people in combination with chemotherapy, after several trials demonstrated superior response rates and survival compared with the cytotoxic agents alone. All evaluated canine unclassified leukemias in this study showed sensitivity to NAV, suggesting that BClxL could be a more effective target in canine leukemia; however, the number of cases assessed in this study was small, and findings must be validated in a larger group of dogs. The clinical utility of BCLxL inhibitors is currently limited by thrombocytopenia because of platelet BCLxL dependence, however development of platelet-sparing BCLxL inhibitors is ongoing.[11,34,35] Given the dismal prognosis of canine AML and ALL after treatment with conventional cytotoxic chemotherapy, further investigation into the role of BH3 mimetics in combination with cytotoxic therapy for dogs with acute leukemia is warranted.[36-38]

This study found that BCL2 protein expression was not consistently associated with VEN sensitivity. Of the two myeloid leukemia samples that showed differential VEN sensitivity, we observed little to no BCL2 protein in the resistant sample, whereas the sensitive sample showed strong BCL2 expression, consistent with findings of the functional assays. In the T cell lymphoma samples, BCL2 protein expression varied from very strong to moderate despite showing a similar VEN EC_{50} between samples (4-5 nM), suggesting that strength of BCL2 expression does not predict sensitivity to VEN. Similarly, in the B cell cancers evaluated, BCL2 expression also varied from low to moderate in VEN-resistant samples. This further supports the notion that detection of BCL2 protein does not predict sensitivity to VEN—a finding which has been reported in the human literature.[32,39,40] In contrast, we found that all canine cell lines and primary samples with little to no BCL2 protein showed resistance to VEN. This suggests that lack of BCL2 expression might be associated with VEN resistance, given cells are unlikely to be BCL2-dependent for survival. This is consistent with reports in the human literature, which have found a lack of VEN sensitivity in cell lines and primary samples with low BCL2 expression.[11,41,42]

We demonstrated that normal lymph node-derived canine B and T lymphocytes are highly sensitive to cell killing by VEN, which is consistent with a previous report of VEN-induced lymphopenia and lymphodepletion in experimentally treated healthy Beagles.[43] There was wide inter-individual variation in sensitivity of normal circulating lymphocytes to VEN, whereas nodal lymphocytes showed more consistent cell killing. The biological explanation for this is unknown but could reflect variable proportions of circulating lymphocyte subsets between dogs, variation between assays, or true heterogeneity of BCL2 protein expression in circulating cells compared with nodal lymphocytes. Indeed, such variation in sensitivity is also observed in circulating lymphocytes in healthy people.[5] Further evaluation in a larger cohort of non-tumor bearing dogs is required.

In vitro sensitivity of primary human CLL samples where the VEN EC_{50} <100 nM are considered sensitive. In people with CLL demonstrating in vitro sensitivity, treatment with daily oral 400 mg VEN results in therapeutic responses.[5,44] Pharmacokinetic studies in people show an area under the curve (AUC) of 31.8 μg hr/mL following 400 mg daily dosing, whereas dogs treated with 20 mg/kg once a day show a comparable AUC of 73.6 μg hr/mL.[43] This suggests that the concentrations required in people to induce cell killing should be achievable in dogs with T-cell cancers. Beagles that received 2 to 20 mg/kg PO daily VEN for up to 9 months developed an average 81% reduction in circulating lymphocytes during this period (including mature B cells and CD4+ and CD8+ T cells), as well as mild anemia; however, no clinical signs associated with leukopenia were noted. Other clinical toxicities reported were grade 2 weight loss associated with hyporexia, and leukotricia.[43] This data suggests VEN could be a safe and well-tolerated treatment for dogs with hematological cancer.

There are several limitations to this study. Only a small number of dogs were enrolled in each group, which increases the possibility for type II error when evaluating associations between lymphocyte or disease subsets and BH3 mimetic sensitivity. Further, phenotyping of samples was limited. Given not all dogs with unclassified leukemia were immunophenotyped by assessment of myeloid and stem cell markers, this might have resulted in a heterogenous population, which could explain the variation in VEN sensitivity observed. Based on clinical and morphological features, acute leukemia was suspected, but

could not be confirmed. To define the role of BH3 mimetics for treatment of acute leukemias, evaluation of a larger group of dogs with more complete immunophenotyping of disease is required. We utilized immunoblotting of bulk cell lysates to assess BCL2 protein. A superior method of protein analysis is flow cytometry, which can distinguish neoplastic cells from normal cells and provide protein quantification based on mean fluorescence intensity. However, the large number of cells needed for the assessment of intracellular protein expression by flow cytometry meant it was impractical for this study, particularly as it is preferable to perform this type of assay on fresh cells. Measurement of mRNA using qRT-PCR was considered, however, given the potential for post-transcriptional modifications that could alter expression, the potential for *BCL2* gene mutations that might alter protein expression, and that our goal was to obtain an overview of BCL2 protein expression amongst different diseases, immunoblotting was considered an appropriate method.[41,45]

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CONFLICT OF INTEREST DECLARATION

Andrew Roberts is an employee of the Walter and Eliza Hall Institute which receives milestone and royalty payments related to venetoclax and receives a share of these royalties from the Institute. Andrew Roberts has received research funding to his institutions from Abbvie for investigator-initiated clinical trials of venetoclax. No other authors have a conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approval by the University of Melbourne Animal Ethics Committee (ID 1914803, approved on April 30, 2019).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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