In vitro Evaluation of ABCB1 After Exposure to Prednisolone and Enrofloxacin in Two Canine Lymphoid Cell Lines: A Pilot Study

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ABSTRACT

Multidrug resistance (MDR), a common cause of chemotherapeutic failure, is associated with the expression of P-glycoprotein (P-gp), encoded for by *ABCB1*, the multidrug resistance gene. Our purpose was to determine whether ABCB1 expression is expressed after exposure of two canine lymphoid cell lines to prednisolone or enrofloxacin. Two established lymphoid cell lines (GL-1, 17-71) were cultured for 14 days, during which cell lines were exposed to drug concentrations equivalent to clinically relevant doses of either prednisolone or enrofloxacin. RNA was extracted from cultured cells and RT-PCR was performed to evaluate *ABCB1* expression.

No *ABCB1* expression was detected in either lymphoid cell line with exposure to prednisolone or enrofloxacin. The *ABCB1* gene does not appear to be upregulated using these drugs in these cell lines in the time frame examined. Pretreatment with corticosteroids prior to chemotherapeutic administration may not induce *ABCB1* drug resistance in canine lymphoid cells as has been reported.

INTRODUCTION

The failure of chemotherapeutic drugs to have an effect on or to produce a response in neoplastic cells has been primarily linked to the development of multiple drug resistance (MDR).[1] Multidrug resistance has been identified as the major obstacle in achieving a

positive clinical response to some of the chemotherapeutic agents used most commonly in both human and veterinary medicine.[2] The development of MDR has a significant impact on both patient and case management, chemotherapeutic drug selection and response to therapy and is associated with a negative response to both the initial therapy as well as to subsequent agents.[3]

Numerous mechanisms of MDR have been identified and some drugs or substrates may achieve resistance through more than one pathway.[3] The most extensively studied and well known proteins involved in MDR development are the ATP-binding cassette transporters, i.e. ABC transporters. They are thought to function normally as a protective mechanism against foreign substances. The most studied ABC transporter is the transmembrane pump, P-glycoprotein (P-gp), a 170 kd phosphoglycoprotein, coded for by the *ABCB1* gene (formerly known as MDR1).[4] In human beings, the negative impact of increased P-gp expression on cancer response rate and remission status has been well documented.[5-8]

Unfortunately, a paucity of information exists in the literature regarding the role and expression of P-gp in canine cancers. Lymphoma is of particular interest as the agents commonly used in its treatment, e.g., doxorubicin, the vinca alkaloids, and glucocorticoids, are known P-gp substrates. In 1995, Moore et al evaluated P-gp expression in canine lymphoma cells prior to chemotherapy and at relapse. As shown by Western blotting, 1 of 30 patients expressed P-gp prior to chemotherapy as compared to 38% at relapse. Based on their results, Moore et al suggested that canine lymphoma could serve as a good model for human non-Hodgkin's lymphoma as it relates to multidrug resistance.[9] In 1996, Lee et al used immunohistochemistry (IHC) on lymph nodes archived from 76 canine lymphoma patients prior to chemotherapy; 33% expressed P-gp. Prospectively, 15 patients were evaluated, and patients in which >50% of the lymphoma cells expressed P-gp at diagnosis had a significantly shorter overall survival time.[10] Lastly, in another study that utilized IHC to detect P-glycoprotein expression in patients with lymphoma at initiation of therapy as well as at relapse and, in some cases, death, frequency of positive staining at initiation was negatively correlated with both remission and overall survival times.[11]

A few studies have evaluated the effect of glucocorticoid exposure on canine cancer. In an osteosarcoma cell line, increased expression of neither P-gp nor another ABC transporter, ABCC1 or MRP1, was detected by Western blotting after 12 weeks of dexamethasone exposure.[12] In contrast, although not all studies concur, evidence exists that prior exposure of canine patients with lymphoma to glucocorticoids is a negative prognostic factor for treatment response.[13-16] In one study, glucocorticoid administration prior to initiation of a complete chemotherapy protocol in canine lymphoma patients resulted in a significantly shorter remission time.[14] However, when evaluating the effect of exposure length, no difference was found in remission between those exposed greater or less than two weeks. Thus, induction of *ABCB1* expression potentially occurred within two weeks of initiation of glucocorticoid therapy, causing resistance to chemotherapeutic agents used. However, to our knowledge, P-gp upregulation by prednisolone or prednisone has not been evaluated in canine lymphoid cells.

Since P-gp substrates may induce pump expression, administration of any substrate can affect chemotherapeutic response. Indeed, the antibiotic doxycycline potentially could affect survival of patients with mammary cancer as it induces P-gp expression in a human mammary carcinoma cell line, MCF-7.[17] Fluoroquinolone antibiotics, however, are more commonly prescribed than doxycycline in veterinary cancer patients. Sparfloxacin and levofloxacin, fluoroquinolones used in human medicine, are known P-gp substrates.[2] Human kidney cell lines and human intestinal Caco-2 cell lines utilize P-gp to remove fluorquinolones.18,19

However, little information is available regarding the relationship between quinolones and ABC transporters in veterinary medicine. Danofloxacin, a veterinary approved fluoroquinolone used in cattle, has been documented to utilize P-gp *in vitro*.[20] To our knowledge, induction of P-gp in canines by the veterinary fluoroquinolone enrofloxacin has not been evaluated. However, the possibilities exist that administration of enrofloxacin could induce multidrug resistance and have long-lasting effects on survival of canine patients with cancer.

Thus, overall, the purpose of our study was to perform a pilot *in vitro* study to determine if exposure of canine lymphoid lines to a glucocorticoid or a fluoroquinolone induces *ABCB1* upregulation. Our first goal was to evaluate the potential for *in vitro* exposure to prednisolone to induce *ABCB1* upregulation or expression in canine lymphoid cells, and, if so, the time-frame of induction. The second goal was to determine if *in vitro* exposure of two canine lymphoid cell lines to enrofloxacin resulted in the upregulation or expression of *ABCB1* over 14 days. Our hypothesis was that the *ABCB1* gene would be expressed or upregulated by 14 days of exposure to either prednisolone or enrofloxacin and that expression would increase over time.

MATERIALS AND METHODS

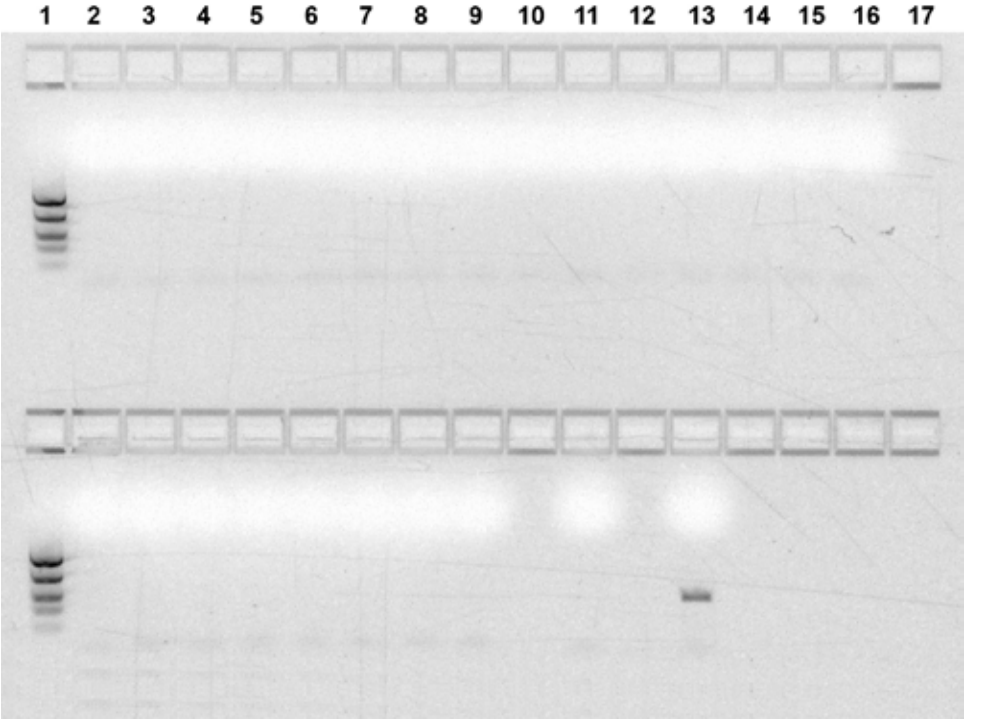
Preliminary study - In a preliminary study, we used two canine neoplastic lymphoid cell lines, GL-1and 17-71, to evaluate cell viability in the presence of prednisolone as well as to establish time points for taking samples.[21,22] The GL-1 cell line was derived from a B cell leukemic canine patient, while the 17-71 cell line was derived from a lymph node of a canine lymphoma patient. GL-1 and 17-71 are commonly used lymphoid cell lines and GL-1 has recently been used shown capable of developing *ABCB1* resistance with continued drug exposure to an *ABCB1* inducing agent.[23-25]

Although culture of the lines used in this project is well-established, we were concerned that the addition of a glucocorticoid might alter cell viability given the effect of glucocorticoids on neoplastic cell survival[24, 26-27] We chose a short timeline (i.e. 72 hrs) because in vivo lung perfusion with doxorubicin in humans induced *ABCB1* expression within 50 minutes.[28] The cells were plated in a 6-well plastic tissue culture plate at a density of 1x105/mL. For the lymphoid cells, the medium was RPMI[a] with 10% fetal bovine serum[b] 1% penicillin/streptomycin/ amphotericin B[c], 1% Glutamax[d] , and 2% sodium bicarbonate[e]. Prednisolone was added to a final concentration of 1.4 µg/ml by diluting a solution of 1000 µg/ml pure prednisolone in methanol. This final concentration was chosen as it represents the mean peak serum concentration achieved in dogs after administering 1 mg/kg prednisolone per os.29 Cells were maintained at 37°C in a 5% CO2 atmosphere. In order to ensure continued drug exposure as would occur with daily dosing in vivo, the medium was changed daily.

Cells from one well were harvested at each time point: 0, 1, 6, 24, 48, and 72 hr. At each time point, 20uL of cells were mixed with an equal volume of trypan blue and were assessed via hemocytometer for cell viability. The remaining cells in the well were placed into 15 mL cylindrical tubes and centrifuged at 1000 rpm for 5 minutes. Excess medium was poured off, 400µL High Pure Lysis Buffer[f] was added to the cell pellet, and the sample was immediately placed at -80°C. For the cells incubated for 48 and 72 hrs, they were evaluated daily microscopically for relative number and integrity and the medium was changed. After 48 hrs, the cells were counted, diluted to a density of $1x10^5$ cells/mL (approximate 1:2 dilution) and replated.

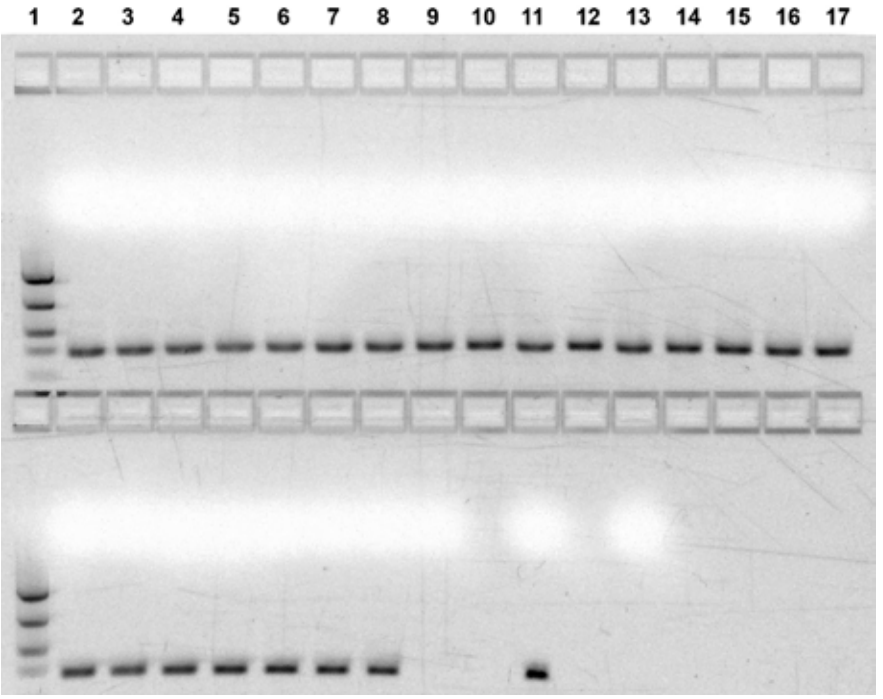
As judged by daily microscopic inspection and trypan blue assays, >98% of cells survived for 72 hours, so all samples were used for analysis. Extraction of RNA from all samples was performed in a single batch. After thawing, the cells were washed once with phosphate-buffered saline. Qiashredder™ [g] devices were used for tissue dis-

Figure 1: *A Gel electrophoresis of ABCB1 PCR products after RT-PCR of RNA isolated from GL-1 cells exposed to prednisolone. With the exception of baseline, samples from each day are arranged with the negative control (cells treated with methanol in media) first followed by the samples from the 3 drug-treated wells.*
Top Row – Lane 1 Ladder; Lanes 2-4 Baseline samples; Lanes 5-8 samples harvested Day 2; Lanes 9-12 samples harvested Day 4; Lanes 13-16 samples harvested Day 7; Lane 17 empty
Bottom Row – Lane 1 Ladder; Lanes 2-5 samples harvested Day 10; Lanes 6-9 samples harvested Day 14; Lane 10 Empty; Lane 11 Negative PCR Control; Lane 12 Empty; Lane 13 Positive PCR Control



ruption and RNA was extracted[f], both per manufacturer's instructions. The quality and quantity of extracted RNA was evaluated spectrophotometrically. Randomly selected samples were further evaluated by gel electrophoresis[h] for RNA band integrity. Reverse transcription was performed using the Biorad iScript™ cDNA Synthesis Kiti per manufacturer's instructions starting with 30 ng RNA for each sample. Polymerase chain reaction (PCR) was performed on the product using previously established primers for canine *ABCB1* and that are not associated with the mutation recognized in certain dog breeds: sense GATTGCTACAGAAGCCATCGAA (bases 2757-2779) and antisense GGGCTGTAG CTGTCAATCAGA (bases 3140-3119).30-32 The thermocycler program was as follows: an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 1 min and then an elongation step of 72°C for 7 min. A PCR was also performed for GAPDH as a housekeeping gene with primers: sense GGCAAATTCCACGGCACAGTCAA and antisense ACATGGGGGCATCAGCAGAAGGA. The PCR protocol was the same as the above *ABCB1* protocol except the annealing temperature was 65°C rather than 57°C. All PCR products were evaluated by gel electrophoresis[h]. Canine hepatocyte cDNA extracted similarly was used as a positive control for *ABCB1* expression.

B – Gel electrophoresis of GAPDH PCR products after RT-PCR of RNA isolated from GL-1 cells exposed to prednisolone. With the exception of baseline, samples from each day are arranged with the negative control (cells treated with methanol in media) first followed by the samples from the 3 drug-treated wells.
Top Row - Lane 1Ladder; Lanes 2-4 Baseline; Lanes 5-8 samples harvested Day 2; Lanes 9-12 samples harvested Day 4; Lanes 13-16 samples harvested Day 7; Lane 17 Day 10 Negative Control;
Bottom Row – Lane 1 Ladder; Lanes 2-4 samples harvested Day 10 Treated cells; Lanes 5-8 samples harvested Day 14; Lane 9 Negative PCR Control; Lane 10 Empty; Lane 11 Positive PCR Control; Lane 12 Empty; Lane 13 Negative cDNA Control; Lane 14-17 Empty

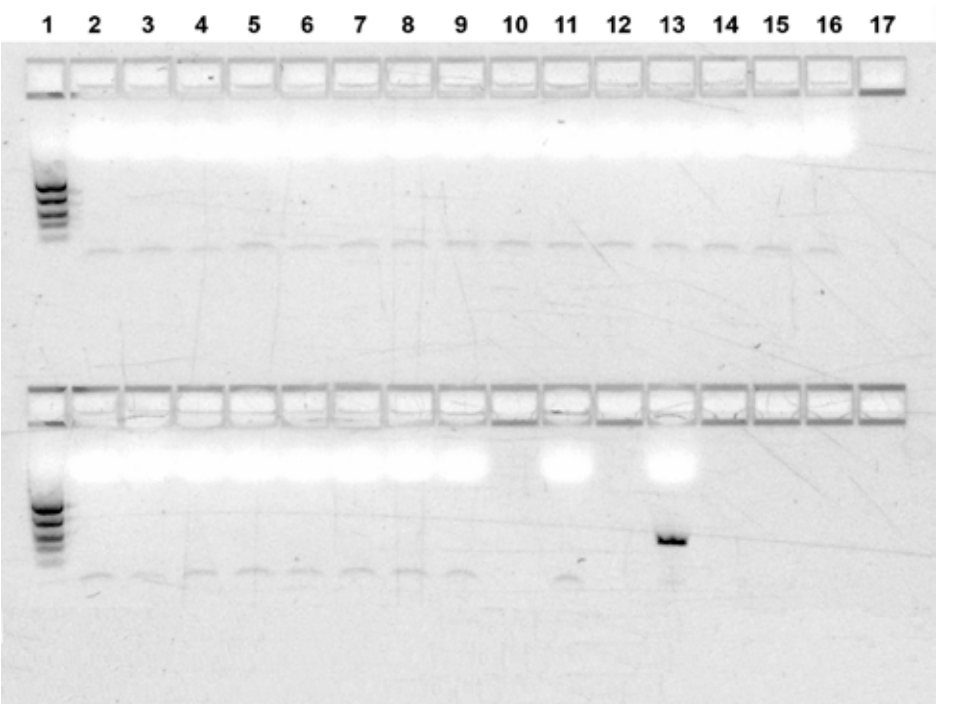


Full study – Based on the preliminary results, a full trial was designed. Cells were cultured and exposed to a negative control (methanol diluent), prednisolone or enrofloxacin for 14 days. For the prednisolone-treated cells, samples were taken for analysis at 0, 2, 4, 7, 10 and 14 days. For the enrofloxacin-treated cells, a sample was harvested after 14 days; the samples harvested at day 0 for the prednisolone-treated cells served as baseline for enrofloxacin treatment. The two lymphoid lines used in the preliminary trial were again employed. Pure enrofloxacin (2000 µg/mL in methanol) was diluted to a target final concentration of 4-6 µg/mL in the medium, a concentration equivalent to the mean peak concentration achieved with once-daily oral dosing at a standard clinical dosing of 10-20 mg/kg of body weight.[33]

The full trial was conducted similarly to the preliminary trial. Trypan blue staining and a cell count were initially performed and the cells were plated at $1x10^5$/mL. The negative control was plated in singlicate and all treated samples in triplicate. Three baseline cell samples were harvested from each cell line (t=0 sample).

Fresh medium was prepared and changed daily in order to ensure ongoing drug exposure as would occur with daily dosing in vivo. Samples were har-

Figure 2 *– Gel electrophoresis of ABCB1 PCR products after RT-PCR of RNA isolated from 17-71 cells exposed to prednisolone. With the exception of the baseline, samples from each day are arranged with the negative control (cells treated with methanol in media) first followed by the samples from the 3 drug-treated wells.*
Top Row – Lane 1 Ladder; Lanes 2-4 Baseline samples; Lanes 5-8 samples harvested Day 2; Lanes 9-12 samples harvested at Day 4; Lanes 13-16 samples harvested on Day 7; Lane 17 empty
Bottom Row – Lane 1 Ladder; Lanes 2-5 samples harvested Day 10; Lanes 6-9 samples harvested on Day 14; Lane 10 Empty; Lane 11 Negative PCR Control; Lane 12 Empty; Lane 13 Positive PCR Control



vested on the designated days. The remaining cells were evaluated daily and cultures were split to a density of $1x10^5$/mL and replated, if needed. Trypan blue staining was performed each time the cells were split. The full trial was performed twice to assess the consistency of the achieved results.

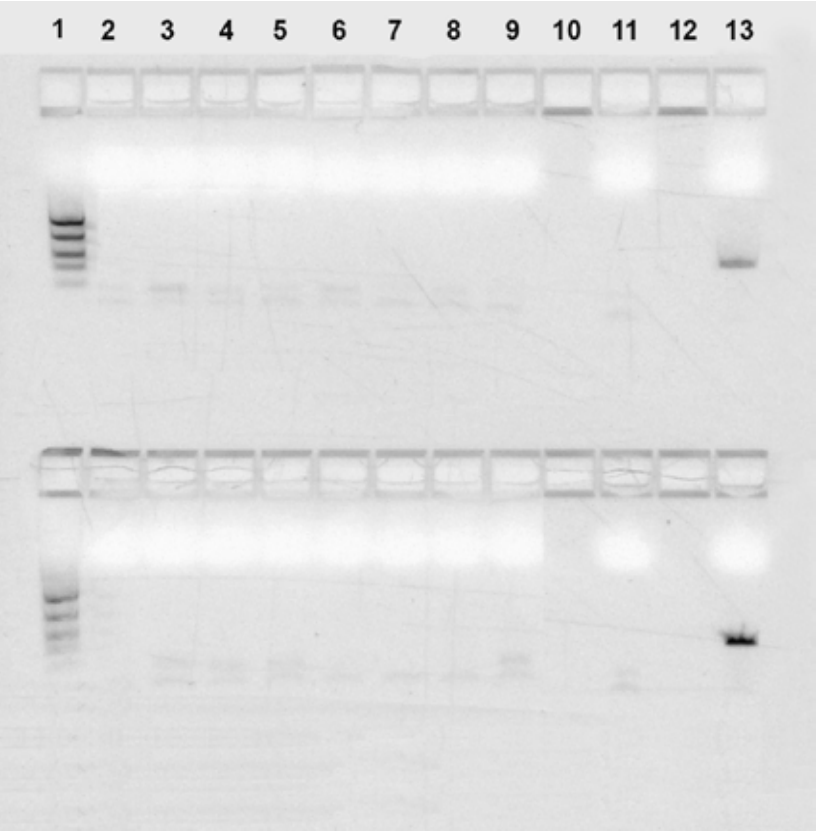
Extraction of RNA, cDNA synthesis, and PCR were performed as in the preliminary trial, except that conditions for the PCR reaction for *ABCB1* were adjusted slightly; the initial denaturation time was increased to five minutes and the final elongation step was increased to ten minutes. All PCR products were evaluated by gel electrophoresis and were the expected length. Representative PCR products were sequenced to verify the product identity.

RESULTS

No *ABCB1* expression was detected during the preliminary trial in either of the lymphoid cell lines at any of the evaluated time points (data not shown). At all time points, cell survival as judged by trypan blue staining was maintained at >98%. Thus, survival of the cells in the presence of prednisolone was demonstrated, at least for 3 days, and the full trial was initiated. *ABCB1* expression was noted in the positive PCR control sample.

For the full trial, given the results of the preliminary trial and a previous publication

Figure 3 *– Gel electrophoresis of Trial 1 and Trial 2 Enrofloxacin treated cells after 14 days exposure, ABCB1 PCR.*
Top Row – Trial 1. Lane 1 Ladder; Lanes 2-5 17-71 cells; Lanes 6-9 GL-1 cells; Lane 10 Empty; Lane 11 Negative Control; Lane 12 Empty; Lane 13 Positive PCR Control
Bottom Row – Trial 2. Lane 1 Ladder; Lanes 2-5 GL-1 cells; Lanes 6-9 17-71 cells; Lane 10 Empty; Lane 11 Negative Control; Lane 12 Empty; Lane 13 Positive PCR Control



suggesting that glucocorticoid exposure may induce *ABCB1* expression in canine lymphoma cells within 14 days, cells were cultured and exposed to the drugs for 14 days.[14] No expression of the *ABCB1* gene was detected after 14 days of exposure to prednisolone in either of the canine lymphoid cell lines, GL-1 and 17-71, in either trial throughout the full study (Figures 1a for GL-1, Figure 2 for 17-71 cells). Expression of the housekeeping gene, GAPDH, was detected in all samples (full trial Figure 1b for GL-1 cells; preliminary trial and full trial for 17-71 cells, data not shown), indicating successful RNA extraction, cDNA synthesis, and amplification of canine *ABCB1* sequence. With regard to enrofloxacin exposure, *ABCB1* expression was not detected after 14 days in either canine lymphoid cell line. (Figure 3)

Both trials yielded the same results for all parameters. In both trials, there was >98% viability of all cells assessed by trypan blue staining.

DISCUSSION

Using established cell lines, we sought to investigate a presumption in the literature that exposure of lymphoma patients to prednisone for two weeks negatively affects survival due to induction of P-gp expression.[14] We used two different lymphoid cell lines as mechanisms of drug resistance may vary between tumor type subsets. Additionally,

as the antibiotic doxycycline induces P-gp expression in a human mammary carcinoma cell line, MCF-7, we studied an antibiotic as well.[17] We chose to include enrofloxacin as it is more commonly used in veterinary cancer patients than doxycycline and certain fluoroquinolones are known P-gp substrates and, thus, could be a P-gp inducer.[2]

The results of our study indicate that 14 days of prednisolone exposure at levels equivalent to clinical plasma concentrations does not induce *ABCB1* gene expression *in vitro* in two canine lymphoid cell lines. Neither did the present study detect induction of *ABCB1* after exposure to enrofloxacin at clinically relevant plasma concentrations for 14 days.

Given our data, the possibility that the chemotherapeutic resistance induced by glucocorticoid exposure in lymphoma patients may not be due to P-gp expression must be considered. First, alternate ABC transporters may be involved. Over 49 human ABC transporters have been identified and classified into seven subfamilies.[2,34,35] Recently, a new variant, MDR3, has been identified in dogs, but the role of this gene has not been established.[36] Second, glucocorticoid pretreatment could induce chemotherapeutic resistance by non-ABC transporter mechanisms, e.g. by altering immunological function. Classically, CD8+ T lymphocytes are central to tumor immunology.[37] Numbers of T lymphocytes are significantly decreased in canine lymphoma and osteosarcoma patients as well as in the lymph nodes of healthy dogs after only three days of oral prednisone administration.[38, 39] With the addition of chemotherapy, patients may not be able to compensate for the immunological dysfunction and exhibit a lack of response to chemotherapeutic agents perceived as resistance.

It is possible that lack of detection of induction of P-gp expression was due to the protocol used. First, cell lines were used, and the results of *in vitro* studies do not necessarily correlate with in vivo results. Unfortunately, in immortal cell lines, the need to initiate or upregulate cell survival mechanisms, such as *ABCB1* expression, may be negated. However, the GL-1 cell line used in this study has been shown capable of inducing an *ABCB1* phenotype in another study. [23]Additionally, the tumor microenvironment in vivo may significantly affect *ABCB1* expression.[40] We attempted to use primary lymphoma cell explants multiple times initially; however, the cells did not survive, most likely due to the cytotoxic effects of steroids and the instability of the cells in culture. Therefore, lymphoid cell lines were utilized to be the best alternative to primary cells. Second, protein expression was not evaluated. Conceivably, P-gp expression could have been present in the lymphoid cells while induction of gene expression was below the sensitivity of the PCR technique. Third, single nucleotide polymorphisms (SNP) have been identified in the canine *ABCB1* gene; the lymphoid cell lines utilized in our study could have contained a SNP leading to lack of PCR primer binding. However, SNP are very unlikely to change PCR primer binding. To our knowledge, the cell lines used in this study are not derived from canine breeds commonly documented to have mutations in the *ABCB1* gene and the primers used in the present study did not span the identified frame shift deletion.

The enrofloxacin and the prednisolone concentrations employed were chosen based upon doses commonly used. Higher drug concentrations could cause upregulation of *ABCB1* gene expression. However, as such concentrations would not be encountered commonly clinically and this was a pilot study, further studies were not performed at this time. Similarly, different glucocorticoids may cause varying responses in P-gp expression.[12,41,42] Indeed, in a prospective study substituting dexamethasone for prednisone in a protocol also incorporating, cyclophosphamide, doxorubicin, and vincristine, to treat canine lymphoma, the median progression- free interval was shorter than in most previous reports in which prednisone was employed; the substitution of dexamethasone for prednisone was suggested to have had a negative impact on survival.[43] Howev-

er, as prednisone is the glucocorticoid used most commonly to treat canine lymphoma we did not study other glucocorticoids.

Use of quantitative reverse PCR (QRT-PCR) is more sensitive than the PCR technique utilized in our study and has been used to evaluate P-glycoprotein expression in canine tissues previously.[44] However, our initial question was whether expression was induced at a clinically applicable dose and duration, the technique we employed provided sufficient information to provide an answer. If changes in expression had been detected, a QRT-PCR assay would have been developed and optimized for further assessment of P-glycoprotein transcript numbers.

CONCLUSION

In conclusion, our pilot study did not provide evidence that glucocorticoid administration before initiation of a chemotherapy protocol affects survival in lymphoma patients by early induction of P-gp expression. Further evaluations into the mechanisms of resistance for canine patients with lymphoma should be pursued as well as further studies similar to this one with other cell lines, primers, and doses. Evaluation of protein expression in further studies would be beneficial to determine if P-glycoprotein expression can be detected even if gene expression is not. Determining which mechanisms of resistance are at play in vivo, would aid in the identification of patients at risk of chemotherapeutic failure, and, potentially, alternative protocol usage with chemotherapeutics that are not P-gp substrates could be utilized in a clinical setting.

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FOOTNOTES

a RPMI 1640 – Invitrogen 61870127 Carlsbad, CA, 92008, USA

b FBS - Fisher Scientific - Pittsburgh, PA 15275, USA

c PSF - Penicillin Streptomycin Solution - Invitrogen 15070063 Carlsbad, CA 92008, USA

d Glutamax - 100X Invitrogen 35050061 Carlsbad, CA, 92008, USA

e Sodium Bicarbonate - Lonza (Biowhittaker) 7.5% Solution 17-613E, CH-4002 Basel, Switzerland

f Roche High Pure RNA Kit – Roche Diagnostics Corporation, Indianapolis, IN,46250-0414, USA

g Qiashredders - Qiagen, Valencia, CA ,91355, USA

h Lonza Flash Gel – Lonza, Basel, Switzerland

i Biorad iScript cDNA Synthesis Kit - Hercules, CA, 94547, USA

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