

***In vitro* drug sensitivity in canine lymphoma**

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Abstract

Introduction: Due to the high heterogeneity of canine lymphoma, the aim of the present study was to test *in vitro* the chemosensitivity of canine high-grade primary lymphoma cells to various cytostatic drugs commonly used to treat dogs: 4-HO-cyclophosphamide, doxorubicin, dexamethasone, prednisolone, vincristine, etoposide, chlorambucil, lomustine, and cytosine arabinoside. **Material and Methods:** To determine the cell viability and drug ability to induce apoptosis two different tests were used: an MTT assay and annexin V/propidium iodide staining. **Results:** Both *in vitro* tests were found to be useful tools. Significant differences in the sensitivity, depending on the drug type, between B-, T- and mixed/null-type lymphoma cells were found for the majority of the tested drugs. B-type cells were the most sensitive *in vitro*, whereas T-type cells seemed to be the most resistant. Doxorubicin, chlorambucil, etoposide, and vincristine most strongly reduced the cell viability and induced apoptosis. **Conclusion:** *In vitro* assays, such as the MTT test and especially the annexin V/PI assay, may be useful tools for predicting a response to the treatment of high-grade lymphoma in dogs or improving the treatment outcomes in individual animals.

Keywords: canine lymphoma, cytostatic drugs, chemosensitivity, MTT test, apoptosis.

Introduction

Lymphoma is the most common haematopoietic malignancy in dogs, constituting up to 83% of cases (11). The main method of treating systemic malignancies like leukaemia and lymphoma is chemotherapy, because it elicits an initial response in the majority of patients. Unfortunately, both normal and cancer cells are sensitive to anticancer agents and this limits the efficacy of conventional cytostatic drugs. The high toxicity of many anticancer substances (particularly bone marrow toxicity) further undermines the effectiveness of the treatment. Another problem involves primary and secondary drug chemoresistance (21).

To maximise the efficacy and reduce adverse effects, chemotherapy is usually based on a combination of drugs with various molecular mechanisms of action

and different side effects. For an effective cancer therapy, the maximal doses should be used that are well-tolerated by a patient without life-threatening consequences. It is also important that each drug has substantial activity as a single agent.

Regardless of the treatment protocol, the majority of canine patients relapse approximately four to nine months after therapy is initiated. Even applying an aggressive therapy to re-induce a remission with rescue protocols often fails and the rate of long-term survival remains poor (10). A lack of pharmacological data for many drugs and a lack of dose escalation studies regarding the use of these drugs in combination may be the reasons for the limited effectiveness of therapy (20).

Due to the marked heterogeneity within each group of cancers and different patient responses to treatment, the therapeutic effects are expected to be

improved by using *ex vivo* drug sensitivity testing. This method enables a clinician to select the drugs with the highest anti-tumour activity or to eliminate the less effective ones (5).

The modified MTT assay (13) was effective in predicting the response to treatment *in vivo* in humans with acute lymphocytic leukaemia (ALL). A correlation was found between *in vitro* and *in vivo* responses to cytosine arabinoside in infants (16), and to prednisolone, vincristine, and L-asparaginase in older children (1, 19).

The aim of the present study was to test the chemosensitivity of canine high-grade primary lymphoma cells (using a modified MTT assay) to the spectrum of cytostatic drugs commonly used in the therapy of dogs, and to compare the results with those obtained from annexin V and propidium iodide staining.

Material and Methods

Sample collection. The material involved dogs with high-grade lymphoma diagnosed at the Clinic of the Diseases of Horses, Dogs, and Cats in the Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Poland (after approval of the local Ethics Committee for Animal Research). In total, 48 lymph node fine needle aspirates (FNAs) were collected from dogs of different breeds, ages, and sexes. Using updated Kiel classification, 31 cases of centroblastic lymphoma of B-cell phenotype, 6 of immunoblastic and 5 of pleomorphic lymphoma of T-cell origin, and 6 cases of pleomorphic lymphoma from mixed/null-cell-type cells were diagnosed.

The classification of immunophenotype was based on the detection of T- and B-cell markers (CD3, CD4, CD8, CD5, CD21, CD14, CD34, CD45, CD45R, CD79 α , and MHC class II) by flow cytometry as described previously (14). When the flow cytometry could not be performed immediately, the samples were fixed with 2% paraformaldehyde and analysed within 2 days. Labelled cells were analysed using a flow cytometer (FACSCalibur, Becton Dickinson, USA). Data were analysed using the Cell Quest 3.1f application (Becton Dickinson, USA). Samples in which the diagnosis was questionable, or where further tests ruled out the malignancy, were excluded from further study. None of the dogs was treated with chemotherapeutics before the analysis.

Cell isolation and cell culture. FNAs from the lymphoma patients were used. Cell suspensions were obtained after several cycles of washing with phosphate-buffered saline (PBS) and erythrocyte lysis (0.84% ammonium chloride, Sigma Aldrich, Germany). The cells were maintained in RPMI 1640 culture medium supplemented with 2 mM of L-glutamine, 100 U/mL of penicillin, 100 μ g/mL of

streptomycin, and 20% of foetal bovine serum (FBS) (all except RPMI 1640 from Sigma Aldrich, Germany). The culture was maintained in a CO₂ incubator at 37°C in a humidified atmosphere.

Reagents. Doxorubicin, dexamethasone, prednisolone, vincristine, etoposide, chlorambucil, lomustine, cytosine arabinoside, FBS, L-glutamine, penicillin, streptomycin, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), *N,N*-dimethylformamide (DMF), and trypan blue solution were purchased from Sigma-Aldrich (Germany). The 4-hydroperoxycyclophosphamide (4-HC) was obtained from Niomech (Germany), dimethyl sulphoxide (DMSO) and ethanol were provided by Avantor Performance Materials, formerly Poch (Poland), and PBS and RPMI 1640 culture medium were obtained from the Institute of Immunology and Experimental Therapy, Wrocław, Poland.

Preparation of drugs. The initial solutions of the tested substances were freshly prepared for each experiment by dissolving in DMSO, ethanol or distilled water. The culture medium was used as a solvent to obtain further solutions.

The compounds were tested at final concentration ranges of 0.005–10 μ g/mL for doxorubicin, 0.1–100 μ g/mL for 4-HC, 0.005–24 μ g/mL for dexamethasone, 0.01–10 μ g/mL for prednisolone, 0.000001–1 μ g/mL for vincristine, 0.005–20 μ g/mL for etoposide, 0.01–20 μ g/mL for chlorambucil, 0.05–50 μ g/mL for lomustine, and 0.01–1 μ g/mL for cytosine arabinoside. The selected range was determined based on the literature for similar tests (5) and on the results obtained using canine cell lines (15).

Determination of cell viability. To determine the cell viability, 2×10^6 cells per well were seeded in a 96-well-plate (Nunc, part of Thermo Fisher Scientific, Denmark). Forty-eight canine lymphoma samples were tested with nine anticancer drugs.

The tested substances were repeatedly diluted with the culture medium (DMSO or ethanol concentration was below 1% in each dilution) and added to the plates with the cells. The cells were incubated for 72 h in a regular culture medium or in a medium containing either the vehicle control or the increasing concentration of the tested substances.

After the incubation, 20 μ L of MTT solution (5 mg/mL) was added to every well. The formazan produced was solubilised after 4 h by adding 80 μ L of the lysis buffer (225 mL of DMF, 67.5 g of SDS, and 275 mL of distilled water). The optical density of the culture wells was measured after 72 h using a spectrophotometric microplate reader (Elx800, BioTek, USA) at a reference wavelength of 570 nm. The viability of test samples was determined as follows: % viability = (average OD for test group/average OD for control group) \times 100. Each test group included four repetitions (four wells each).

Detection of apoptosis. Early apoptosis was detected using the Annexin V-FITC Apoptosis

Detection Kit (Sigma-Aldrich, Germany), according to the manufacturer's instructions.

Briefly, the cells were treated with etoposide, lomustine, prednisolone, or 4-HO-cyclophosphamide at a final concentration of 10 µg/mL, chlorambucil at 5 µg/mL, dexamethasone at 24 µg/mL, vincristine at 1 µg/mL, cytosine arabinoside at 1 µg/mL, or doxorubicin at 0.5 µg/mL for 24 h. The concentration of the test compounds used was chosen based on the results of the MTT viability test, carried out for a wide range of concentrations.

After harvesting, the cells were washed twice with cold PBS, suspended in a binding buffer, and stained with annexin V-FITC and propidium iodide for 10 min at room temperature in the dark. After the incubation, flow cytometric analysis was immediately performed using a FACSCalibur flow cytometer (Becton Dickinson, USA). CellQuest 3.1f software (Becton Dickinson, USA) was used for data analysis based on dot plots of FL1-H and FL2-H, showing the population of dead cells and the cells undergoing phosphatidylserine externalisation.

Statistical analysis. The ANOVA test was used in order to determine the differences between three groups of canine lymphoma (B-, T-, and mixed/null-cell-type), for each drug. Tukey's post-hoc test was used to indicate significantly different groups. The statistical level of significance was set at $P < 0.05$. All calculations were performed using STATISTICA v.10 (StatSoft, Poland).

Results

Determination of cell viability. Viability of the cells evaluated by trypan blue exclusion assay at the time of setting the test ranged from 90% to 98%. Microscopic examination of the cells after 72 h of incubation revealed noticeable reduction of cell viability in the subject and control wells, manifested by the presence of dead cells or cells exhibiting characteristic apoptotic features (blebbings).

Lymphoma cells of T-phenotype were characterised by a greater viability than the neoplastic B-cells. In general, cells isolated from the canine patients did not exhibit satisfactory proliferative potential *in vitro*, which may be associated with the lack of essential growth factors in the culture medium.

Significant differences in the sensitivity of B-, T-, and mixed/null-type lymphoma cells to different drug types were found. Vincristine reduced the cell viability most strongly in all the cell types (the percentage of viable cells after incubation with 1 µg/mL was 17.1 ± 5.8 for B, 35.1 ± 6.2 for mix/null-type cells, and 19.8 ± 8.7 for T-cells). The drug with the weakest activity against all tested types of tumour cells was lomustine (average viability of cells, after incubation with 10 µg/mL of the drug, was 62%). Glucocorticoids (dexamethasone and prednisolone) were characterised

by comparable potency but B-type cells revealed the highest sensitivity to this group of drugs. Prednisolone (10 µg/mL) reduced cell viability of B-type lymphoma to $25.2 \pm 9.6\%$ and to $39.9 \pm 6.7\%$ and $56.6 \pm 9.5\%$ of mixed/null- and T-cell-type respectively. The differences between B- and T-types were statistically significant ($P < 0.05$).

Among alkylating agents (chlorambucil, 4-HC, and lomustine), chlorambucil showed the strongest activity. The most sensitive to these substances were B- and mixed/null-type cells. Chlorambucil at the dose of 5 µg/mL reduced cell viability to $16 \pm 7.2\%$ and $24.3 \pm 5.4\%$ respectively, while T-cells were much more resistant ($42.3 \pm 9.8\%$ of viable cells).

Cytosine arabinoside was the representative of antimetabolites, whose mechanism of action is to interfere with the DNA synthesis through a competitive antagonism with normal nucleotides. This drug has demonstrated a moderate efficiency at the concentration of 1 µg/mL, in particular with regard to the B-cells ($64.5 \pm 10.5\%$ of viable cells), but also was effective against mixed/null-type cells ($65.2 \pm 9.6\%$ of viable cells). T-cells were more resistant ($92.4 \pm 9.8\%$ of viable cells) and it was statistically significant in comparison with B-cells.

Doxorubicin (anthracycline), which acts by inhibiting the synthesis of RNA, was tested as an anticancer antibiotic. High efficacy of the drug against all cell types was demonstrated. B- and mixed/null cells were most sensitive (the percentages of viability after incubation with 0.5 µg/mL were $13.9 \pm 11.2\%$ and $17.8 \pm 6.1\%$ respectively). Etoposide (exerting its action mainly through the influence on the topoisomerase II) affected all lymphoma/leukaemia cell types strongly (the percentages of viable cells were $22.1 \pm 7.8\%$ for B-, $32.5 \pm 10.1\%$ for mixed/null-type, and $40.6 \pm 7.2\%$ for T-cells).

The cell viability after 72 h of incubation with three different drugs is shown in Fig. 1.

Apoptosis assay. The percentage of apoptotic cells was determined simultaneously with the MTT assay. As mentioned earlier, the cell viability, examined using trypan blue exclusion assay, ranged from 90% to 98%. Studies with annexin V and propidium iodide allowed not only determination of the ability of the tested substance to induce apoptosis, but also specification of the percentage of alive and apoptotic cells in the untreated samples. This test confirmed the cellular death in the control wells also observed under the microscope.

The numbers of live cells in the control group after 24 h of incubation averaged $52.3 \pm 16\%$ for B-cell, $72.7 \pm 6\%$ for T-cell, and $55.5 \pm 8\%$ for mixed/null-cell-type lymphomas. Moreover, it was shown that under *in vitro* conditions, B-cells spontaneously underwent apoptosis and the number of apoptotic cells in the control sample correlated with the increased sensitivity to the drugs (Fig. 2).

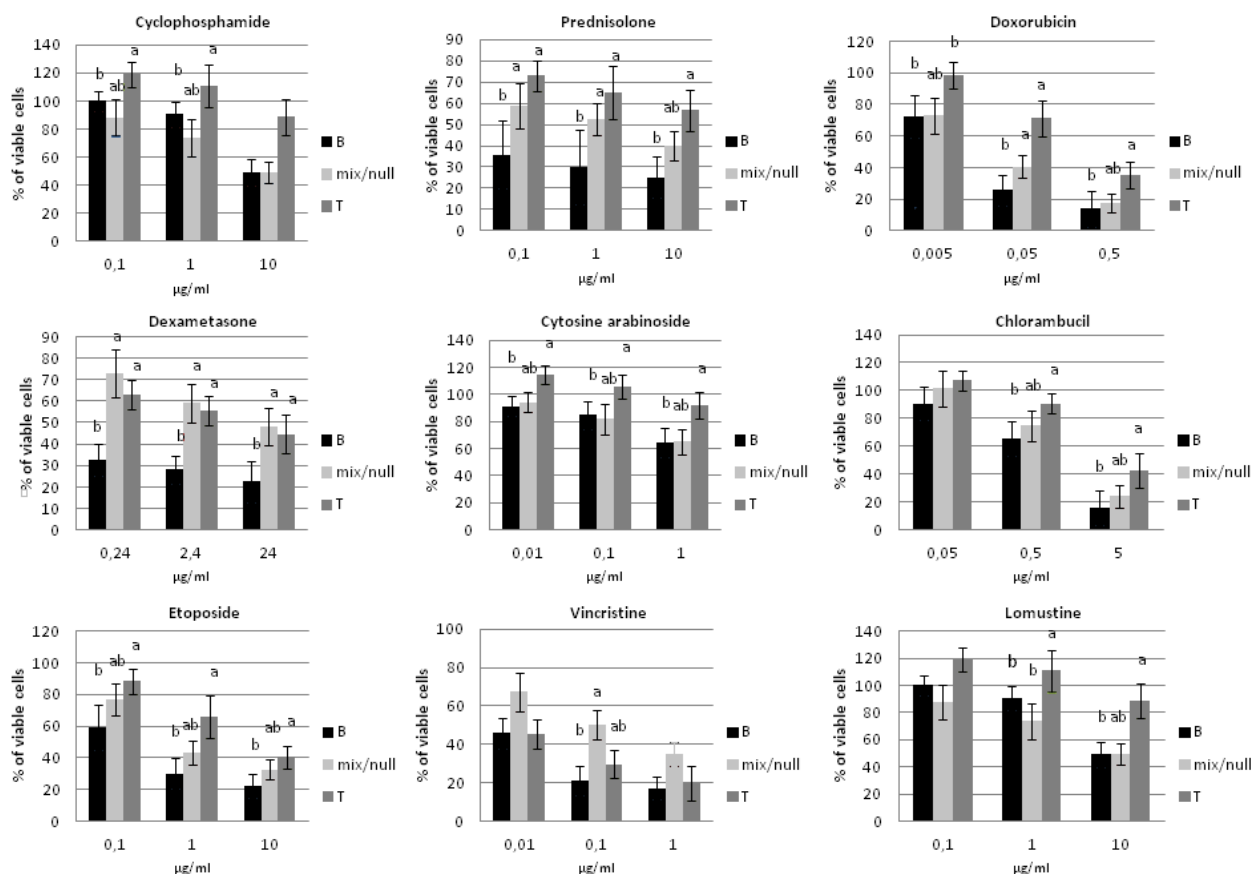


Fig. 1. Percentage of viable B, T, and mixed/null-type cells after 72 h of incubation with three different concentrations of 4-HC, prednisolone, doxorubicin, dexamethasone, cytosine arabinoside, chlorambucil, etoposide, vincristine, and lomustine evaluated with MTT assay. For each concentration of the drug, values without common letters (a, b, ab) in the superscript differ statistically ($P < 0.05$). Results are presented as averages \pm the standard deviations of at least four independent experiments

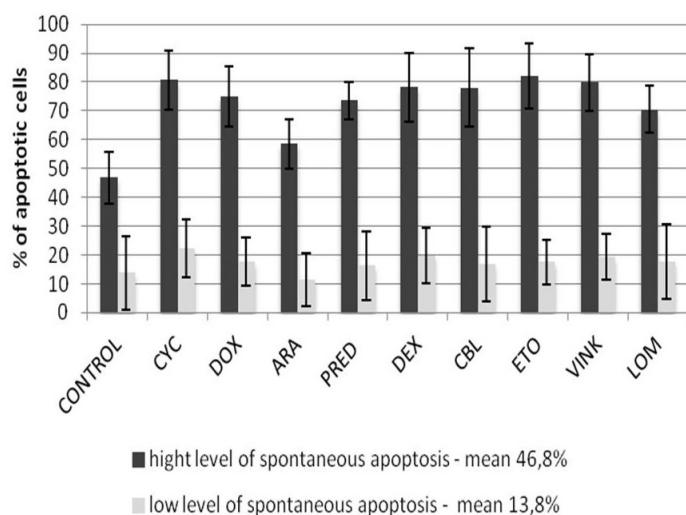


Fig. 2. Percentage of apoptotic cells after 24 h of incubation with 4-hydroperoxycyclophosphamide (CYC), etoposide (ETO), lomustine (LOM), prednisolone (PRED), all at a final concentration of 10 $\mu\text{g/mL}$, chlorambucil (CBL) at 5 $\mu\text{g/mL}$, dexamethasone (DEX) at 24 $\mu\text{g/mL}$, vincristine (VINK) and cytosine arabinoside (ARA) at 1 $\mu\text{g/mL}$ or doxorubicin (DOX) at 0.5 $\mu\text{g/mL}$, depending on the number of cells undergoing apoptosis spontaneously. The percentage of apoptotic cells was much larger in samples with a high level of spontaneous apoptosis (mean 46.8% of apoptotic cells in the control). Results are presented as averages \pm the standard deviations of at least three independent experiments

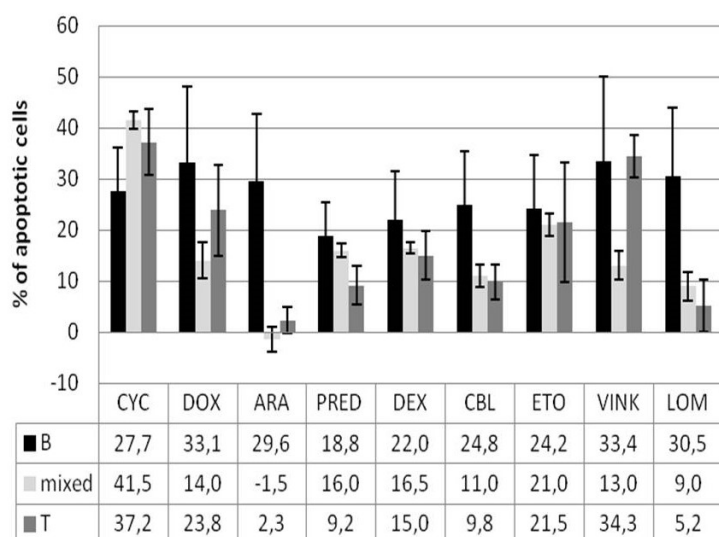


Fig. 3. Percentage of apoptotic cells after 24 h of incubation with 4-hydroperoxycyclophosphamide (CYC), etoposide (ETO), lomustine (LOM), prednisolone (PRED), all at a final concentration of 10 $\mu\text{g/mL}$, chlorambucil (CBL) at 5 $\mu\text{g/mL}$, dexamethasone (DEX) at 24 $\mu\text{g/mL}$, vincristine (VINK) and cytosine arabinoside (ARA) at 1 $\mu\text{g/mL}$ or doxorubicin (DOX) at 0.5 $\mu\text{g/mL}$. The percentage of apoptotic cells after the incubation with the drug was obtained by subtracting the percentage of apoptotic cells in the control from each test point. Results are presented as averages \pm the standard deviations of at least four independent experiments

In the samples in which a high level of spontaneous apoptosis was observed, higher sensitivity of cells to the tested substances was also found.

The 4-HC, etoposide, and vincristine were the strongest apoptotic triggers in all cell types, and they were also the most effective in the MTT assay. B-type lymphoma cells were the most sensitive to all the tested substances except 4-HC, while T-type cells were the most resistant to the majority of tested compounds (Fig. 3). These results correlated with the ones obtained using the MTT assay (Fig. 1).

Discussion

Many different cancer cell lines are used to investigate therapeutic methods and cancer biology. However, chemosensitivity studies carried out on established cell lines do not always reflect the actual patient situation (18). Therefore, apart from cell-line-based studies, primary culture studies predominate as they better reflect the actual type of malignant cells and their sensitivity to drugs. This is particularly important in the heterogeneous types of cancer, such as lymphoma.

It is currently known that each individual tumour may be genotypically and phenotypically different and, therefore, may require the use of different drugs (3). In addition, the cells obtained from a patient often display a multi-drug-resistant (MDR) phenotype, which may be the cause of a therapy failure (21).

For this reason, many tests have been developed to assess drug resistance in tumour cells that allow

selection of the most effective treatment protocol (2). When carrying out such a test, two obstacles can be encountered: obtaining a sufficient number of cells and the difficulties associated with their culture. The sample contamination with normal cells characterised by different (in most cases weaker) sensitivity to cytostatic drugs is another problem (8).

To evaluate the viability of the cells after incubation with a panel of anticancer drugs, a considerable amount of cells is required ($2 \times 10^6/\text{mL}$). This is due to the limited proliferative potential of canine lymphoma cells *in vitro*. A rich culture medium or the use of a layer of feeder cells expressing the CD40 ligand or CD40 ligand provided in soluble two-trimeric forms, are useful (7).

Despite the limited proliferative activity of canine lymphoma cells *in vitro*, the MTT assay was found to be a useful tool for determining the sensitivity profiles of commonly-used cytotoxic drugs. However, the waiting time for the test results (72 h), in addition to the significant amount of cells required for the study, is a fundamental drawback. This study showed that the annexin V/PI assay required a smaller amount of cells and the results were known after 24 h.

Although the drugs commonly used in the treatment of lymphoma in dogs are not typical compounds inducing apoptosis, the test with annexin V/PI enables evaluation of not only the amount of apoptotic cells, but also the number of living and dead cells. In this study, we found a correlation between the cells' ability to undergo spontaneous apoptosis and their sensitivity to the cytostatic properties of the tested drugs. Kitada *et al.* (9) drew a similar conclusion that

the amount of B-CLL cells, spontaneously entering apoptosis, did not correlate with the expression of any individual apoptosis-regulatory proteins.

In our study, T-cells exhibited a significantly lower sensitivity to the majority of tested substances than B-cells. These results correlate with a clinical investigation showing poorer prognosis in the case of high-grade T-cell canine lymphoma (6), and with the results of similar studies involving the use of established canine lymphoma and leukaemia cell lines (15). The use of drugs with proven *in vitro* activity against specific tumour cells may be useful in solving this problem. The greatest difference in the sensitivity between different cells was observed in the case of steroidal anti-inflammatory drugs. They are widely used in the treatment of lymphomas and leukaemias in dogs, particularly due to their availability, safety, and relatively low price. As a part of complex anticancer protocols, glucocorticosteroids show the expected therapeutic efficacy, particularly when used in B-cell lymphomas. In the absence of the possibility of use of another treatment, monotherapy with glucocorticosteroids for T-cell lymphoma or leukaemia should be connected with a worse prognosis than in the case of B-cell neoplasms. It was also found that primary-cultured B-cells, collected from dogs with lymphoma and leukaemia, were sensitive *in vitro* to steroidal anti-inflammatory drugs, which are clearly different from some canine lymphoma and leukaemia cell lines like GL-1(15).

In our research T-cells showed a weaker sensitivity to the steroidal anti-inflammatory drugs. Much attention is paid to a worse response to treatment in patients treated with steroids prior to the relevant chemotherapy. This phenomenon is not fully understood (17). An activated B-cell diffuse large B-cell lymphoma (ABC-DLBCL) is an aggressive, poorly chemoresponsive lymphoid malignancy. It is characterised by constitutive canonical NFκB activity that promotes lymphomagenesis and chemotherapy resistance *via* an overexpression of antiapoptotic NFκB target genes. Inhibition of the canonical NFκB pathway may therefore prove relevant in ABC-DLBCL treatment (4). Matsuda *et al.* (12) showed that inhibition of NFκB transcription factor restored the glucocorticoid receptor expression and the sensitivity of CL-1 and GL-1 cells to the effects of dexamethasone.

Knowing the degree of individual tumour cell sensitivity to glucocorticoids, one could try to modify the pharmacotherapy in order to achieve a satisfactory therapeutic effect. To avoid the aforementioned resistance in the treatment of lymphomas, a fast diagnosis (through the use of simple and rapid diagnostic techniques) and an appropriate application of the selected therapeutic programme (without prior use of steroidal anti-inflammatory drugs) are required.

In vitro assays, such as the MTT and especially the annexin V/PI, may be useful for predicting the

response to treatment of lymphoma in dogs. Chemosensitivity tests, molecular biology, and pharmacogenomic techniques can significantly contribute to improving the treatment outcomes for individual canine patients. The use of additional growth factors supporting proliferation of the cells *in vitro* certainly allow more tests to be performed.

The improvement can be made by eliminating the drug with the lowest efficiency or by modifying therapies that increase the sensitivity of cells to the used substances. Further studies are especially needed on the correlation of *in vitro* test results with clinical data and patient response to treatment.

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