Summary of the Ph.D. thesis

# INVESTIGATION OF IMMUNOPHENOTYPE AND CHEMOTHERAPY RESISTANCE IN MOUSE AND CANINE LYMPHOMA/LEUKEMIA CELLS

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# 1. Introduction and aims of the study

## 1.1 Introduction

Non-Hodgkin's lymphoma (NHL) is one of the most common neoplasia among human and canine patient. Approx. 5300/100,000 dog population rate are diagnosed with cancer in the USA which is 10 times more than among human population where the annual incidence is approx. 500 per 100,000. However, there are similarities not only in incidence rates between human and canine NHL, but also in genetic and biological behaviour, and in the relationship between the development of the disease and various infectious and environmental factors. Spontaneously occurring tumours that are equivalent morphologically and clinically in humans and dogs allowing for comparative studies to understand the pathogenesis of this disease.

Analysing subtypes there are also similarities and some differences. In both species diffuse large B-cell lymphoma (DLBCL) is the most common subtype, but in dog the next common subtype is the Peripheral T-cell Lymphoma Not Otherwise Specified (PTCL-NOS) which is rare among human patients. In veterinary practice, 60% to 80% of canine lymphomas are B-cell origin in which response rates to chemotherapy is better, the relapse free

period (RFP) and overall survival time (OST) is longer than in the 10% to 38% occurring T-cell lymphomas. For accurate determination of immunophenotype, there are different methods like immunohistochemistry (IHC), cytology, flow cytometry (FC) or even PCR for Antigen Receptor Rearrangements (PARR). In veterinary practice, immunohistochemistry, using indirect staining, became widespread as a general diagnostic method. However, in cancer therapy, an important factor is to make an early diagnosis and start the right therapy as soon as possible, in which FC can help for veterinarians. Measurement with FC is faster by the use of directly labelled monoclonal antibodies and less invasive tumour sampling method is needed using fine needle aspiration (FNA) technique. In addition, beside the differential diagnosis of hematopoietic malignancies (as immunophenotyping, staging, detecting minimal residual disease), flow cytometry can be used for a number of other purposes, such as functional measurement of the cell surface P-glycoprotein efflux transporter (Pgp). Prognostic role of Pgp detected by immunohistochemistry in canine lymphoma was investigated in several studies, but the relationship with survival time was not clear. Detection of Pgp can also be performed by FC. Calcein

assay is a suitable method to measure Pgp activities. The value, derived from the measurement, is the Multidrug resistance Activity Factor (MAF). In the case of human lymphoma MAF was found to be a prognostic factor in line with the therapy response. However, this measurement was not investigated in canine tumour samples.

Lymphoma is considered a systemic disease that affects lymphocytes and therefore systemic therapy is the first line treatment. In untreated dogs, survival time is only 4 to 6 weeks, while in order to achieve remission (RFP: 238 days) and prolong survival time (OST: 344 days) intensive chemotherapy is needed. Multiagent therapy is more effective than single-agent therapies. Although there are several different protocols for the treatment of lymphoma, most are based on the so-called CHOP (cyclophosphamide [C], doxorubicin [H, hydroxydaunorubicin], vincristine [O, Oncovin] and prednisone [P]) protocol, which is also used for human disease. With this therapy, remission can be achieved in 80-90% of canine cases, quality of life can be improved and the median survival time can be increased to 10-12 months. However, acquired resistance is often observed against initially effective treatment agents. Several mechanisms may be responsible for the development of

multidrug resistance (MDR), but the most common cause is a membrane component with a molecular weight of 170 kDa. Pgp is a protein, member of the ABC family, that is capable to transport substrates through biological membranes using ATP hydrolysis. Pgp can recognize and eliminate a wide variety of drugs, including cytostatic agents (vinca alkaloids, anthracyclines, taxanes). Three of the treatment agents of the mentioned CHOP protocol are Pgp substrates, therefore the efflux pumps in tumour cells are able to transport these agents to the extracellular space, thereby reducing the intracellular drug concentration.

Within 10 years of identifying the role of ABC transporters in the mechanism of drug resistance, research focused on Pgp and other ABC transporter inhibitors. Pgp can be inhibited and 3 generations of inhibitors can be distinguished, however, the clinical benefit is still questionable. Due to the essential physiological protective role of Pgp (on various barriers, intestinal tract, kidney, liver), its inhibition resulted a number of unexpected side effects.

There are various alternative strategies against Pgp such as monoclonal antibodies or active immunization or even gene silencing and the use of

tyrosine kinase inhibitors (TKIs). In addition to various inhibitors, cyclooxygenase-2 (COX-2) enzyme was discovered that also affects Pgp activity. Activation of COX-2 / PGE2 / PGE receptor signaling pathway enhances not only MDR1 gene encoding Pgp, but also MRP1 and BCRP, which play an important role in MDR development. However, the use of various COX-2 inhibitors as non-steroidal anti-inflammatory drugs (NSAIDs) may have the potential to inhibit the expression of transporter proteins, thereby enhancing the cytotoxic effect of the antitumor agent without generating more toxic side effects. Firstly, in 2002, a U.S. research team was able to demonstrate the direct link between COX-2 and Pgp activity. The increase in MDR1 mRNA expression depended on the level of COX-2 expression. It was then proved that the COX-2 inhibitor also reduced Pgp expression and activity by flow cytometry and Western blot analysis. Since then, the association was confirmed in several cell lines and tumour types. Furthermore, increased COX-2 expression can also be observed in canine lymphoid tissues, however, the use of selective COX-2 inhibitors to overcome drug resistance was not studied among canine patients.

## 1.2 Aims of the study

1. In the present study, our aim was to compare the methods of immunophenotyping, the advantages and disadvantages of flow cytometry in contrast to immunohistochemistry. Furthermore, another aim was to determine whether the measurement of Pgp activity can be performed by flow cytometry as a part of routine diagnostic method at the time of diagnosis. Our long-term goal was to compare the initial resistance status with Pgp activity measured during chemotherapy treatment in dogs, which may give some information about the role of Pgp in the development of the therapy resistance. According to these information, patient tailored therapy can be used by choosing the suitable treatment agents.

2. Our further aim was to investigate the change in the efficacy of veterinary therapeutic agents depending on Pgp expressession in tumour cells. For this reason, a drug-sensitive and resistant pair of *in vitro* mouse leukemia cell line and a diffuse large B-cell canine lymphoma cell line were used.

3. Another aim was to create an *in vitro* model system to study the development of drug resistance in which

sensitive tumour cells become resistant against treatment like observed during canine patient therapy. This system would modelise similar drug resistance during cytostatic therapy used in dogs.

4. Our main goal was to use Pgp inhibitors (either epigenetic or others, such as COX-2) in our *in vitro* model system that can be useful agents in canine lymphoma therapy. Thus, doxorubicin was combined with various epigenetic inhibitors [temozolomide, trichostatin-A (TSA), and SAHA] and COX-2 inhibitors (celecoxib, firocoxib, meloxicam, mavacoxib). In this *in vitro* experiment, combined treatments were investigated whether Pgp activity, which is responsible for the development of therapy resistance, can be inhibited.

## 2. Materials and methods

### 2.1 Investigation of methods for immunophenotyping

Our study, where the different methods for immunophenotyping (IHC, FC) were compared, involved 35 dogs diagnosed with multicentric lymphoma, 17 females and 18 males, their average age was 8 years. In a T-cell rich B-cell lymphoma case study the two diagnostic methods were also compared. The patient in

the case study was an 11-year-old female Yorkshire Terrier dog treated according to the CHOP protocol.

To determine the immunophenotype, the tumour sampling was performed during surgery or by fine needle aspiration technique which is similar to the cytology sampling. After the surgery, entire lymph node sample was taken into 10% neutral buffered formalin and was sent to an external laboratory for further processing. For fine needle aspiration sampling, an 18G needle was used for the flow cytometry and a 22G needle for the cytology. The sample was placed in a tube containing supplemented RPMI (Life Technologies, Carlsbad, USA) or on a slide. Then, further processing was performed according to different diagnostic methods.

During immunohistochemistry, lymph node sample was fixed and then paraffin-embedded sections were used. To stain T and B-lymphocytes different polyclonal antibody KITs were used. Following CD3, CD79a / CD20 staining, secondary antibodies were used with DAB (3,3'-Diaminobenzidine) staining as described in the protocol. Hematoxylin Gill II (Merck KgaA, Darmstadt, Germany) was used for background staining. Dog tonsilla tissue was used as positive control, and the labeling protocol without primary antibody was used for the

negative control. To determine the percentage of each CD markers the expressing areas of best quality for both stains were selected and 200 cells per field were counted in 5 high-power fields.

For flow cytometry analysis lymph node samples were immersed into dissociation medium containing collagenase and dispase, and after a 30-minute-long incubation at 37°C, cells were separated by a 40-µm cell strainer to create cell suspension. $10^6$ cells were stained with direct labelling monoclonal antibodies, CD3, CD21 and their isotype control. After a 30-minute-long incubation at 37°C, staining was stopped with 1mL ice-cold PBS and cells were centrifuged at 300 g, finally cells were measured in 270 µL PBS by FACScan. To evaluate the percentages of CD3 and CD21 markers, BD CellQuest (Becton Dickinson, San Jose, USA) statistical program was used. Finally for cytology after alcohol fixation was used and both slides were stained with quick staining Panoptic kit.

## 2.2 Functional Pgp analysis in canine lymphoma

The study included 12 dogs whose Pgp activity was compared with at the time of diagnosis and during the therapy from the recurrent tumour. There were 4 females,

8 males and their average age was 6.8 years. In 2 additional cases, Pgp activity was measured four times from the diagnosis to the end of therapy. In case 1, a 3.5-year-old male German Shepherd was diagnosed with large cell immunoblastic lymphoma, stage V. (substage 'b'). Patient 2 was a 6.5-year-old female, cocker spaniel, dog, diagnosed with DLBCL, stage IV. (substage 'a').

Following sampling, calcein assay was used to measure P-glycoprotein function. The essence of the method is that the non-fluorescent Calcein AM dye converted into a highly fluorescent dye by intracellular esterase enzymes, then the fluorescent intensity can be measured by flow cytometry. However, the P-glycoprotein inhibits the accumulation of Calcein AM (0.25 mmol/L) in the cells, thereby reduced fluorescent intensity can be measured in Pgp-positive cells. As Pgp inhibitor verapamil (10 mmol/L) is added to the cells, the signal intensity significantly increases, so the function of Pgp can be well monitored in different cells. 250,000 cells per group were used for this assay. MAF was calculated from the mean of histograms measured in the presence (mean fluorescence inhibited (MFI)) or absence (non-inhibited (MFNI)) of verapamil, using the following formula: MAF = (MFI - MFNI)/MFI.

To determine the relationship between the two methods for immunophenotyping (IHC, FC) Pearson's correlation analysis (Microsoft Excel 2019, Microsoft, Washington, USA) was used. The $p < 0.05$ was considered as statistically significant.

### 2.3 Investigation *in vitro* mouse and canine lymphoma cell lines for testing different drugs

For *in vitro* studies, P388 mouse B-lymphoblastic leukemia cell line, its doxorubicin-resistant pair, P388/ADR cells and CLBL-1 canine diffuse large B-cell lymphoma suspension cell line were cultured in RPMI (Roswell Park Memorial Institute) media (Life Technologies) supplemented with 10% fetal bovine serum, 5 mmol/L glutamine, and 50 units/mL penicillin and streptomycin (Life Technologies). All cell lines were cultured at 37°C with 5% $CO_2$. To test the cytotoxicity of drugs, cells were seeded into 96-well tissue culture plates and treated with compounds for 120 hours. Following incubation time, PrestoBlue® assay was added and cells were measured spectrophotometrically using an EnSpire microplate reader (Perkin Elmer).

To further investigate the role of Pgp, we also measured the efficacy of doxorubicin and its special form,

pegylated liposomal doxorubicin (PLD), in a mouse allograft model. The animal experiments were performed in the Department of Experimental Pharmacology at National Institute of Oncology in compliance with the necessary permits (22.1 / 2291/3/2010) and with the EU regulations for animal testing. $10^6$ P388 and P388/ADR cells were injected intraperitoneally into 6-8 weeks old BDF1 mice. After 48 hours a single dose of saline, doxorubicin (3 mg/kg) or PLD (3 or 5 mg/kg) were administered intraperitoneally. Following treatment, mice were weighted 3 times per week and monitored daily (stress, discomfort, pain) using the Body Condition Scoring (BCS) method.

In the following tests, mRNA expression of those genes (Abcb1a, Abcb1b), which are responsible for the development of resistance, was measured on drug-sensitive and resistant cells during doxorubicin monotherapy. P388 cells were homogenized in TRIzol™ Reagent (Life Technologies) then total RNA was isolated using Direct-zol® MiniPrep kit (Zymo Research) according to the manufacturer’s instructions. 300 ng total RNA was reverse transcribed to cDNA using the Promega Reverse Transcription System Kit. For real-time PCR assay, Beta-Actin (Aktß) probe (Life Technologies) was

used as an endogenous control, while the expression of mouse Abcb1a and Abcb1b genes was quantified with the appropriate TaqMan® primers on a StepOne™ Real-Time PCR instrument (Life Technologies). The mRNA fold changes were determined by the $2^{-\Delta\Delta Ct}$ method.

### 2.4 Creating an *in vitro* model system to study resistance in lymphoma cells

In most cases, the concentration that killed 20% of the cells was used ($IC_{80}$). However, in the case of doxorubicin, an $IC_{10}$ concentration was used, i.e. 90% of the cells were killed by the treatment. Based on these, P388 cells ($10^6$ cells) were treated with 13 nM doxorubicin in a T75 suspension flask for 120 hours, or doxorubicin was supplemented with celecoxib (16 μM), firocoxib (16 μM), trichostatin-A (30 nM) or SAHA (0.4 μM) in the combined treatment to investigate how Pgp expression can influence the efficacy of different treatments. In the experimental design, following the 5-days-long mono or combined therapy, cells were cultured in a drug-free medium, which was changed in every 5 days until surviving cells reached the initial $10^6$/18 mL density (repopulation time). Treatments were repeated several times, after every third treatment MAF was determined

using the calcein assay, and the sensitivity of the tumour cells to chemotherapeutic agents was measured by cytotoxicity assay.

This experimental design was also used in canine B-cell lymphoma (CLBL-1) cells, but the initial density in this case was $10^7$ cells. The following concentrations were used for each drug: DOX (0.3 nM), trichostatin-A (50 nM), SAHA (0.7 µM), celecoxib (26 µM), firocoxib (26 µM), meloxicam (20 µM) and mavacoxib (40 µM).

### 2.5 Combination of doxorubicin with COX-2 and epigenetic inhibitors already used in veterinary medicine in mouse and canine lymphoma cell lines to prevent the development of resistance

The drug interaction of combined treatments was also examined in a short-term 5-day measurement. Mouse and canine cells were seeded into 384-well plates. The combination of treatment agents was added to the cells by a Hamilton StarLet automated liquid handling system. Incubation was at 37 °C in 5% $CO_2$. $GI_{50}$ (Growth Inhibition) values were determined using PrestoBlue® reagent (Life Technologies) and measured with an EnSpire microplate reader (Perkin Elmer). $GI_{50}$ values of ‘compound 1’ with the fixed concentrations of ‘compound

2’ (and vice versa) were paired, and plotted on an equipotent graph as $GI_{50}$ isoboles. For each data point of the isobole, significance was calculated as the combination index (CI) based on Chou’s study (2006).

Statistical analyses were performed using the GraphPad Prism 8 software; growth curve of cytotoxicity assays and the Kaplan-Meier survival curves was carried out with this program. One-way or two-way ANOVA followed by Tukey’s multiple test was used for comparisons between treatment groups and MAF values for each cell line. The $p < 0.05$ was considered as statistically significant.

## 3. Results and discussion

### 3.1 Investigation of methods for immunophenotyping

In the study for comparing immunohistochemistry (IHC) and flow cytometry (FC) among 35 lymphoma samples, a strong significant correlation was found between the percentage of B and T cell markers ($R_{CD21}$ = 0.6560 ($p = 0.00002$), $R_{CD3}$ = 0.8334 ($p < 0.00001$), respectively. Furthermore, in a lymphoma case study, the expression of CD20 marker was 43%, but CD3 positivity was also detected. Based on the expression pattern of CD markers and morphology of the cells, the diagnosis was

T-cell rich B-cell lymphoma. This case was also analysed by FC, which showed that 62% of larger cells were positive for B-cell marker and 14% for T-cell marker. However, analysing smaller cells, this ratio changed to 33:47%. The results of FC measurement were supplemented by cytological examination thereby the same diagnosis was identified as with IHC. Our conclusion: FC is a suitable diagnostic method for lymphoma immunophenotyping. The method is faster, fine needle aspiration is relatively non-invasive sampling technique compared to IHC where surgical excision is needed but morphological analysis can only be performed upon IHC. FC measurement should be evaluated together with cytology to have morphological data. In the case of IHC fewer cells are analysed, but the FC software can detect 10,000 cells, however, the IHC method should not be supplemented with further tests. These two methods cannot be considered as fully substitutable tests.

### 3.2 Functional Pgp analysis in canine lymphoma

Examining another prognostic factor for lymphoma, MAF was determined in 12 dogs. In 3 cases, the MAF showed an increased value already at the time of diagnosis (mean 0.33 ± 0.03), but during the

chemotherapy, these values increased even higher, reaching a mean value of 0.47 (± 0.1). With the other 9 dogs, the mean MAF value was only 0.08 (± 0.07) at diagnosis, but it increased significantly using therapy, mean value reached 0.38 (± 0.13). The median survival time of the patients was 396 (± 179) days, which was consistent with data reported in other studies. However, the period from the onset of relapse was only an average of 120 (± 104) days. We also demonstrated that the flow cytometer is also suitable for functional measurement of Pgp. The transporter that appears in tumour cells is often responsible for the development of multidrug resistance (MDR), which inhibits the long-term efficacy of chemotherapeutic agents and affects survival time.

Similar process was detected in the following study, where resistance status was measured four times with 2 canine patients thereby the development of therapy resistance was more observed. The study showed that the immunophenotype, identified at diagnosis, remained the same during CHOP treatment (CD3-, CD5-, CD11 / 18-, CD14-, CD21 +, CD34-, CD45 +, MHCII +). In contrast, the change in MAF value was continuous using the treatment. In case 1, lack of P-glycoprotein activity was indicated (MAF = 0.01) at the time of diagnosis.

However, as a result of CHOP therapy, the MAF value increased continuously, reaching 0.56 by the end, when the size of the lymph node became increased. In case 2, significant Pgp activity could be detected at the time of diagnosis (MAF = 0.35), possibly because of prednisolone pre-treatment, and then increased to 0.52 after 7 additional treatments. From day 136, due to financial reasons, the treatment was temporarily halted for 33 days, this period was called drug holiday (DH). This unplanned drug holiday resulted in a significant decrease in Pgp activity (MAF = 0.22). However, following two additional cycles of treatment, tumour cells regained Pgp expression (MAF = 0.31) and the size of the lymph node also increased. The immunophenotype of the lymphoma remained identical during the therapy.

Our studies showed that MDR can develop as a result of treatment (acquired resistance) or may appear at the beginning of treatment (intrinsic resistance). However, in both cases, it influences the effectiveness of treatment. Although the importance of the Pgp was identified with an unfavorable therapeutic response among patients in other studies, but this was the first time when flow cytometry was used for measuring Pgp activity in canine tumour samples.

### 3.3 Investigation *in vitro* mouse and canine lymphoma cell lines for testing different drugs

After demonstrating the role of Pgp in the development of therapy resistance *in vivo*, the effect of treatment agents was also investigated *in vitro*. With P388 cells MAF was 0.09, no Pgp activity was observed in tumour cells, compared to resistant P388/ADR cells, where the MAF was 0.97. The difference was also detected by cytotoxicity assay in which the $IC_{50}$ values were compared and P388/ADR cells survived 13,600-fold more toxicity than P388 sensitive cells. A Pgp inhibitor, tariquidar (TQ), was added to the doxorubicin treatment in P388/ADR cells thereby the DOX sensitivity was closer to P388 untreated cells and the difference reduced to 4.6-fold. By this method, Pgp-mediated resistant was evidenced in P388/ADR cells, because in the absence of inhibitor, Pgp could recognize and pump out DOX. Following this test, a special form of doxorubicin, pegylated liposomal doxorubicin (PLD), was also investigated. Similar results were found, there was a 13,587-fold difference in PLD sensitivity between P388 and P388/ADR cells. However, in the presence of Pgp inhibitor, this reduced to 3.14-fold.

PLD tests were also performed in a mouse allograft model. PLD causes fewer side effects and could therefore be given at a higher dose than doxorubicin, so doses of 3 and 5 mg/kg were also used. Drug-sensitive P388 cells had better therapy response for both DOX and PLD treatments, with a significant increase in survival time (DOX: 29 days; PLD 3 mg/kg: 28 days; PLD 5 mg/kg:> 63 days) compared to the saline-treated group (15.5 days). Using the same treatments, the survival time could not be extended in the resistant cells (DOX: 12.5 days; PLD 3 mg/kg: 13 days; PLD 5 mg/kg: 16 days).

The role of Pgp was also demonstrated *in vitro* that it could influence the efficacy of treatments. The results of the allograft model were consistent with *in vitro* data showing that DOX and PLD were not effective in Pgp-mediated resistant tumour cells despite higher doses.

Next step was to test different veterinary drugs that can be potential treatment agents for lymphoma. To perform this test, firstly the $IC_{50}$ concentrations were determined *in vitro* in P388 mouse cells, but temozolomide was not toxic, only at very high concentration ($IC_{50}$ = 430 μM), and in the case of SAHA or TSA, the concentrations which killed 50% of the cells, were 0.56 μM and 41 nM.

To study canine lymphoma, the panel was expanded with a more relevant cell line and compounds were also tested on the CLBL-1 diffuse large B-cell canine lymphoma cell line. CLBL-1 naive cells expressed low levels of Pgp, MAF was 0.22. Sensitivity of various chemotherapeutic agents and epigenetic inhibitors was also tested in CLBL-1 cell line. The $IC_{50}$ of DOX was 1 nM, while the $IC_{50}$ of SAHA was 0.76 µM, it was 57 nM with TSA and in the case of temozolomide it was 80 µM.

All in all, the P388 and P388/ADR mouse cell lines were good pair for studying mechanism of resistance, while CLBL-1 is a more relevant model for canine lymphoma.

### 3.4 Creating an *in vitro* model system to study resistance in lymphoma cells

In the following *in vitro* study, our aim was to model what we observed in the clinic. In a new *in vitro* model system, drug-sensitive P388 cells were treated for 5 days with one of the compounds of the CHOP protocol, doxorubicin at $IC_{10}$ concentration, and then the surviving cells were cultured further in drug-free medium (repopulation). In the case of P388, the DOX $IC_{10}$ concentration proved to be effective even after second

treatment, when the number of surviving cells decreased. However, the effect was no longer observed after the third treatment, number of cells increased despite therapy, and Pgp expression (MAF = 0.6) increased significantly after 42 days. Similar result was measured with canine B-cell lymphoma cell line. Parental CLBL-1 cells were treated with 0.3 nM DOX, which was effective for up to 5 treatments. Despite the sixth treatment, tumour cells began to grow indefinitely and became resistant to DOX, within 74 days of treatment Pgp activity significantly increased (MAF = 0.42). Therapy response, increased MAF value together with the cytotoxicity assay results clearly showed that the initial P388 and CLBL-1 cells were nine times more sensitive compared to DOX treated cells (P388 D and CLBL-1 D). Using tariquidar TQ, Pgp-mediated resistance was also evidenced in these experiments.

According to our clinical observation, the unplanned drug holiday was an option for temporarily overcome resistance, resulting in a decrease ratio of Pgp-positive tumour cells. After modelling the development of the resistance, the effect of drug holiday was also investigated in these cells. For this reason, previously DOX-treated resistant P388 D and CLBL-1 D tumour cells

were cultured in drug-free medium, thereby P388 D/DH cells showed decrease in MAF after 32 days (MAF = 0.47), while MAF value of CLBL-1 cells decreased to 0.26 from 0.42 after 27 days drug holiday. Cytotoxicity assay also confirmed that the 9-fold difference between resistant and untreated cells was reduced to 3.6-fold ($p < 0.0001$) in P388 cells due to drug holiday and it was only 2-fold in CLBL-1 cells.

In parallel with the functional and cytotoxicity measurements, mRNA expression levels of those genes, which were responsible for the development of resistance, were also determined in the following study. Expression levels of the mouse Abcb1a and Abcb1b genes were significantly increased by DOX treatment, while a significant decrease was observed in expression level of Abcb1a gene in P388 cells after drug holiday.

In conclusion, the change of resistance status was observed in clinical practice *in vivo* and it was proved *in vitro*, too. In our model system, tumour cells became resistant against treatment in a similar way as in canine lymphoma patients. This model seems to be more relevant for studying resistance, than using cells such as P388/ADR which was created for almost 1 year under continuous selection pressure.

### 3.5 Combination of doxorubicin with COX-2 and epigenetic inhibitors already used in veterinary medicine in mouse and canine lymphoma cell lines to prevent the development of resistance

In our *in vitro* model system, the experimental design was modified by using different epigenetic inhibitors (TSA, SAHA) simultaneously with DOX treatment. After every third treatment, MAF values were measured (MAF 1, 2, 3) and the period while the MAF remained lower than 0.2 was detected. Cells were considered resistant above the cut-off value. Examining this period, there was no significant difference between DOX+TSA (40 days) and DOX+SAHA (41 days) compared to DOX monotherapy (30 days). After 9 treatments, the MAF value increased significantly in the P388 treatment groups (MAF≥0.6), but also in CLBL-1 cells MAF values were higher than 0.2 after the 6th treatment.

In the following experiments, the effects on Pgp activity were investigated with the combination of DOX and different COX-2 inhibitors (celecoxib, firocoxib, meloxicam, mavacoxib). At first, $IC_{50}$ concentrations of the inhibitors were determined in both cell lines, however, it

was soon revealed that firocoxib and mavacoxib were not toxic in mouse and canine tumour cells even at high concentrations.

In long-term combination experiment the period, during MAF remained under 0.2 cut-off value, was compared which was the following in different treatment groups: 36 days for DOX treatment, 44 days for DOX+mavacoxib, 51 days for DOX+meloxicam, 67 days for DOX+firocoxib. However, surprisingly, using DOX+celecoxib combination, Pgp activity of the tumour cells did not reach 0.2 MAF after 9 treatments (for 100 days) ($p = 0.0073$). A significant ($p < 0.05$) difference was observed in MAF values after the 6th and the 9th DOX+CEL treatments compared to DOX monotherapy. In this latter case Pgp activity showed significant increase after 3 treatments only, in P388 cells. The results of the combination of COX-2 inhibitors were similar in CLBL-1 cells, while Pgp activity in groups such as DOX+mavacoxib (MAF = 0.39), DOX+meloxicam (MAF = 0.36) and DOX+firocoxib (MAF = 0.29) treated cells was close to DOX treated cells. However, the most effective combination was the DOX+CEL, too, and significant ($p < 0.05$) difference was measured after 9 cycles of treatment compared to DOX monotherapy.

Our studies showed that although the combination therapy with HDAC inhibitors was not significantly more effective than doxorubicin alone, but chemotherapy combined with celecoxib could inhibit the development of Pgp induction.

After the *in vitro* long-term combination experiment, in a short-term, 5-days assay, the interaction between the previously used treatment agents (epigenetic and COX-2 inhibitors) and DOX was investigated. An antagonistic effect was measured between DOX and celecoxib and between DOX and meloxicam, thereby these therapeutic agents did not increase their toxicity in P388 and CLBL-1 cells. The effect was also antagonistic between DOX and firocoxib, but this was observed only in CLBL-1 cells and in P388 cells using DOX and TSA. In contrast, there was a synergistic effect between DOX and firocoxib on the P388 cell line and between DOX+TSA in CLBL-1 cells.

In the following experiment, the drug sensitivity was measured by cytotoxicity assay. There was no significant difference in the sensitivity of doxorubicin between untreated and DOX+CEL treated cells in both cell lines.

Our previous study revealed that inhibition of Pgp expression was not the result of synergistic toxicity of DOX and CEL, so in the next *in vitro* experiment, we examined whether celecoxib can exploit a change in the cells after DOX treatment. In this experiment, DOX and CEL were not used simultaneously, but after a 5-day DOX pre-treatment, the cells were continuously treated with the COX-2 inhibitor, while the control group was cultured in drug-free medium. Examining the number of surviving cells, 50% of P388 cells died during celecoxib treatment, while CLBL-1 tumour cells did not survive this treatment which otherwise kill only 20% of tumour cells thereby P388 and CLBL-1 became more sensitive after DOX pre-treatment.

In the next experiment, we examined whether CEL can inhibit Pgp function in a doxorubicin-resistant cell line. Due to the increased Pgp activity, low calcein intensity was measured in cells, however, high fluorescence intensity was observed when Pgp inhibitor, verapamil was added to the cells. Celecoxib was used in the same concentration as in the model system and its 10-fold dose. There was no difference between the histograms, so celecoxib was not able to cause direct Pgp inhibition. In the next step, MAF measurements were performed after

one month of drug holiday or celecoxib treatment. After 28 days DH, MAF decreased from 0.97 to 0.82 in P388/ADR cells as a result of drug holiday, but using celecoxib treatment further reduction was detected in Pgp activity (MAF = 0.74). A similar result was measured in CLBL-1 canine tumour cells, where the MAF value of resistant cells changed from 0.36 to 0.28 due to the 28-day drug-free period, while this change was even greater with celecoxib (MAF = 0,19). It is suggested that some of the cells lost Pgp transporter activity.

Based on these results a randomized double-blind controlled study was initiated in the Veterinary Hematology and Oncology Clinic where the relapse free and overall survival time of lymphoma patients is measured in the standard CHOP treated group and in the new modified CHOP+CEL treated group.

## 4. New scientific results

1. We proved that lymphoma immunophenotyping determined by flow cytometry and combined with cytology strongly correlated with the results of immunohistochemistry, however, the FC is a faster, and less invasive method in veterinary oncology.

2. The routine diagnostic use of Multidrug Resistance Activity Factor (MAF) allowed us to easily measure and confirm the development of Pgp-induced resistance caused by cytostatic therapy among dogs diagnosed with lymphoma, which often results the failure of the treatment.
3. In two canine lymphoma case studies, it was possible to examine the change of Pgp activity at several sampling times during chemotherapy and we could observe that this process was reversible due to the drug holiday in case 2.
4. We created an *in vitro* model system in which tumour cells became resistant due to a stepwise selection method not under the classical continuous selection pressure. We developed resistant tumour cells, mouse and canine types, in a similar way as we use chemotherapeutic agents in clinical practice. This provided the opportunity to study resistance *in vitro* and look for new strategies to solve this problem.
5. We also demonstrated *in vitro* that the development of resistance is reversible and drug holiday can temporarily reduce Pgp activity.

6. In our COX-2 inhibitor panel (celecoxib, firocoxib, meloxicam, mavacoxib), we could prove that celecoxib combined with doxorubicin could prevent the development of MDR in both mouse and canine lymphoma cells. Furthermore, we were able to reduce Pgp activity in the resistant tumour cells with celecoxib treatment. We found that this is not a direct effect on inhibition of Pgp and is not achieved by increasing the toxicity of doxorubicin.

## 5. Publications based on the results of the doctoral study

### 5.1 Publications in peer-reviewed scientific journals with impact factors

Karai E., Dékay V., és Vajdovich P.: **Az áramlási citométer, mint a lymphoma diagnosztikájában alkalmazható eszköz az állatorvosi onkológiában**, MÁL, 142. 531–544, 2020.; IF (2019): 0.107

Karai E., Szebényi K., Windt T., Fehér S., Szendi E., Dékay V., Vajdovich P., Szakács G. és Füredi A.: **Celecoxib Prevents Doxorubicin-Induced Multidrug**

**Resistance in Canine and Mouse Lymphoma Cell Lines**, Cancers, 12(5). 1117, 2020.; IF (2020): 6.162

Füredi A., Szebényi K., Tóth S., Cserepes M., Hámori L., Nagy V., Karai E., Vajdovich P., Imre, T., Szabó P., Szüts D., Tóvári J. és Szakács G.: **Pegylated liposomal formulation of doxorubicin overcomes drug resistance in a genetically engineered mouse model of breast cancer**, Journal of Controlled Release, 261. 287–296, 2017.; IF (2017): 7.877

Dékay V.; Karai E.; Szakács G.; Füredi A.; Szebényi K.; Vajdovich P.: **Calcein Assay for Multidrug Resistance Predicts Therapy Response and Survival Rate in Canine Lymphoma Patients**, in Preparation. 2020.

## 5.2 International conference presentations

Edina Karai, András Füredi, Gergely Szakács, Péter Vajdovich: **Significance of the epigenetic regulation to inhibit the development of chemotherapy resistance**, Budapest Breast Think Tank Conference, 2016.

Edina Karai, András Füredi, Kornélia Szebényi, Gergely Szakács, Péter Vajdovich: **Significance of the epigenetic regulation to inhibit the development of chemotherapy resistance**, 1st Veterinary Oncology and Clinical Pathology Meeting Visegrád, 2016.

Edina Karai, András Füredi, Kornélia Szebényi, Gergely Szakács, Péter Vajdovich: **Significance of the planned drug holiday in canine lymphoma treatment**, Veterinary Oncology and Clinical Pathology Congress, Nantes, 2016.

Edina Karai, András Füredi, Kornélia Szebényi, Gergely Szakács, Péter Vajdovich: **Possibilities to inhibit the development of canine chemotherapy resistance**, ESVONC Congress Lyon, 2017.

Edina Karai, Eszter Szendi, Kornélia Szebényi, András Füredi, Barbara Rütgen, Tímea Windt, Péter Vajdovich: ***In vitro* model system for the emergence of chemotherapy resistance with CLBL-1 cell line**, ESVONC Congress Las Palmas de Gran Canaria, 2018.

Edina Karai, András Füredi, Gergely Szakács, Kornélia Szebényi, Barbara Rütgen, Péter Vajdovich: **COX-2 inhibitors to overcome drug resistance on canine lymphoma cell line**, 2019 VCS Annual Conference, Houston, 2019.

## 5.3 National conference presentations

Karai Edina, Füredi András, Szakács Gergely, Vajdovich Péter: **Kutya limfómák drog rezisztenciájának kialakulása**, Akadémiai beszámolók, Klinikumok, 2015.

Füredi András, Szebényi Kornélia, Karai Edina, Vajdovich Péter, Szakács Gergely: **Drog rezisztencia kutyák, egerek és emberek daganataiban**, Akadémiai beszámolók, Klinikumok, 2015.

Karai Edina, Füredi András, Szakács Gergely és Vajdovich Péter: **Kutya-lymphomák drog-rezisztenciájának vizsgálata,** Akadémiai beszámolók, Klinikumok, 2016.

Dékay Valéria, Karai Edina, Verebélyi Tamás, Koltai Zsófia, Vajdovich Péter: **A multidrogrezisztencia fehérje-1 (MDR1) immunhisztokémiai és funkcionális vizsgálatának összehasonlítása lymphomás, emlődaganatos és mastocytomás kutyák vizsgálata során**, Akadémiai beszámolók, Klinikumok, 2016.

Karai Edina, Füredi András, Szebényi Kornélia, Vajdovich Péter és Szakács Gergely: **Az epigenetikai szabályozás jelentősége a kemoterápia rezisztencia kifejlődésének megakadályozásában**, 46. Membrán-transzport Konferencia Sümeg, 2016.

Karai Edina, Füredi András, Szebényi Kornélia, Szakács Gergely és Vajdovich Péter: **Lehetőségek a kutya kemoterápia rezisztencia kialakulásának megakadályozására**, Akadémiai beszámolók, Klinikumok, 2017.

Karai Edina, Connor Herst és Vajdovich Péter: **Áramlás citometriai analízis validálása kutya tumorok immunfenotipizálása során**, Akadémiai beszámolók, Klinikumok, 2018.

Karai Edina, Windt Tímea, Füredi András, Szebényi Kornélia, Szakács Gergely és Vajdovich Péter: **Kemoterápiás vegyületek együttes hatásának vizsgálata egér és kutya lymphoma sejtvonalakon**, Akadémiai beszámolók, Klinikumok, 2018.

Karai Edina, Füredi András, Szebényi Kornélia, Szakács Gergely és Vajdovich Péter: **Kombinációs kemoterápia alkalmazása egér és kutya lymphoma sejtvonalon**, Akadémiai beszámolók, Klinikumok, 2019.

Karai Edina, Füredi András, Szebényi Kornélia, Windt Tímea, Kucsma Nóra, Szakács Gergely és Vajdovich Péter: **A celecoxib hatásvizsgálata monoterápiában és doxorubicinnel kombinálva egér és kutya lymphoma sejtvonalon**, Akadémiai beszámolók, Klinikumok, 2020.