Multi-drug resistance in a canine lymphoid cell line due to increased P-glycoprotein expression, a potential model for drug-resistant canine lymphoma

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Canine lymphoma is routinely treated with a doxorubicin-based multidrug chemotherapy protocol, and although treatment is initially successful, tumor recurrence is common and associated with therapy resistance. Active efflux of chemotherapeutic agents by transporter proteins of the ATP-Binding Cassette superfamily forms an effective cellular defense mechanism and a high expression of these transporters is frequently observed in chemotherapy-resistant tumors in both humans and dogs.

In this study we describe the ABC-transporter expression in a canine lymphoid cell line and a sub-cell line with acquired drug resistance following prolonged exposure to doxorubicin. This sub-cell line was more resistant to doxorubicin and vincristine, but not to prednisolone, and had a highly increased P-glycoprotein (P-gp/abcb1) expression and transport capacity for the P-gp model-substrate rhodamine123. Both resistance to doxorubicin and vincristine, and rhodamine123 transport capacity were fully reversed by the P-gp inhibitor PSC833. No changes were observed in the expression and function of the ABC-transporters MRP-1 and BCRP.

It is concluded that GL-40 cells represent a useful model for studying P-gp dependent drug resistance in canine lymphoid neoplasia, and that this model can be used for screening substances as potential P-gp substrates and their capacity to modulate P-gp mediated drug resistance.

1. Introduction

Canine lymphoma (cL), the most common hematopoietic neoplasia in the dog, is in many respects comparable to non-Hodgkin lymphoma in humans (Teske, 1994). As in humans, treatment in the dog consists of a multidrug chemotherapy protocol that includes, as a minimum, cyclophosphamide, doxorubicin, vincristine and prednisolone (or CHOP-protocol) (Garrett et al., 2002; Sorenmo et al., 2010) of which, as a single agent, doxorubicin appears most effective (Simon et al., 2008). Despite a high initial response rate, tumor relapse is common and more often refractory to therapy, leading to treatment failure and ultimately the dog's death (Flory et al., 2011; Zandvliet et al., 2013). The main cause for treatment failure is thought to be tumor drug resistance (DR) that can be present at the start of chemotherapy (intrinsic DR) or develop during or following chemotherapy (acquired DR). Several clinical studies on cL (Price et al., 1991; Piek et al., 1999; Gavazza et al., 2008; Marconato et al., 2011) have documented that treatment with glucocorticoids prior to starting chemotherapy lowers the overall response rate and shortens survival and this has been explained by assuming acquired, glucocorticoid-induced DR (Mealey et al., 2003).

One of the mechanisms underlying DR is the active efflux of (cytotoxic) drugs by membrane bound transporter proteins of the ATP-Binding Cassette (ABC) superfamily (Gottesman, 2002; Huang et al., 2004). High expression of these ABC-transporters, and P-glycoprotein (P-gp; ABCB1) in particular, has been associated with both a decreased sensitivity to cytotoxic agents, as well as a poor prognosis in several types of cancer in humans (Huang et al., 2004; Gottesman et al., 2002; Kourti et al., 2007; Efferth et al., 2008) and dogs (Bergman et al., 1996; Lee et al., 1996; Steingold et al., 1998; Honscha et al., 2009). Other ABC-transporters associated with DR to cytotoxic agents in humans include multidrug resistance related protein 1 (MRP1; ABCC1) and breast cancer resistance protein (BCRP; ABCG2) (Gillet et al., 2007), both of which have been studied in veterinary medicine (Honscha et al., 2009; Tashbaeva et al., 2007; Schrickx and Fink-Gremmels, 2008; Mealey, 2012), but not in relation to cL.
Although DR represents a major obstacle in the successful management of cancer with chemotherapy in both humans and dogs, therapeutic measures to circumvent DR are still limited (Szakacs et al., 2006). Studying the mechanisms responsible for DR will provide a better understanding of DR (Chen and Sikic, 2012) and could potentially lead to the development of new therapies (Efferth et al., 2008; Zhu et al., 2009). For the dog both these goals would be greatly facilitated with a canine in vitro model, but a previously reported DR cell line (Uozurmi et al., 2005) derived from the canine lymphoid GL-1 cell line (Nakaichi et al., 1996) is no longer available.

The first goal of the current study was to re-establish a DR canine lymphoid cell line through selection for doxorubicin-resistance. Doxorubicin was purposefully chosen given its’ efficacy in the treatment of CL, but also because resistance to doxorubicin is predictive for multidrug resistance in human neoplasia (Efferth et al., 2008). The second goal was to assess in both the original and the doxorubicin resistant sub-cell line, the antiproliferative effect of the drugs used in a CHOP-protocol, and the expression and function of the ABC-transporters P-gp, MRP1 and BCRP in both cell lines.

2. Material and methods

2.1. Chemicals

PSC833 (Valspoda®) was a gift from Novartis Pharma AG (Bazel, Switzerland) and Ko143 was kindly provided by Prof. Koomen (University of Amsterdam, the Netherlands). MK571 sodium salt was obtained from Alexis Biochemicals (Grünberg, Germany). Rhodamine 123 (Rh123), and 5(6)-carboxyfluorescein diacetate (CFDA) were purchased from Sigma–Aldrich (St Louis, MO, USA). Pheophorbide A (PhA) was obtained from Frontier Scientific (Logan, USA). Doxorubicin hydrochloride and vincristine sulfate and prednisolone were purchased from Sigma–Aldrich (St Louis, MO, USA).

2.2. Cell lines, cell culture media and supplements

The canine lymphoid cell-line, GL-1 cells (Nakaichi et al., 1996), was kindly provided by dr K. Ohno (University of Tokyo). The cells were confirmed to be of canine origin through DNA-sequencing (four regions, 900 base-pairs in total) and showed 100% homology with the canine reference genome. GL-1 cells grow in single cell suspension and showed strong immunoreactivity with CD34 and CD79α, a weak reaction with CD3 and CD4 and no reaction with CD21 and CD90, consistent with a precursor lymphoid cell of the B-lineage.

GL-1 cells were grown in suspension on RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 1% (v/v) l-glutamine (BioWhittaker, Maine, USA), 10% (v/v) fetal bovine serum (Gibco) and 100 U/ml penicillin and 100 μg/ml streptomycin (BioWhittaker) at 37 °C, 5% CO2. The GL-1 cells were cultured with gradually increasing concentrations of doxorubicin and after 6 months a subline of the GL-1 cells was selected that could be cultured in the presence of 0.07 μM (40 ng/mL, hence GL-40) doxorubicin. Multiple batches of these GL-40 cells were stored in liquid nitrogen and were maintained in culture after thawing following a schedule of one passage with doxorubicin (20 ng/mL) and two passages without doxorubicin.

2.3. Proliferation assay

Cell proliferation was assessed with the Cell Counting Kit-8 assay (CCK-8, Dojindo Molecular Technologies, Rockville, Maryland, USA) using the tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye following bioreduction in the presence of the electron carrier, 1-methoxy PMS. Cells were seeded in 96-well plates at a density of 2 × 104 cells per well in cell culture medium containing a concentration range of doxorubicin, vincristine or prednisolone and incubated for 24, 47, and 72 h at 37 °C, 5% CO2. The tetrazolium solution was added to each well 2½ h before light absorbance analysis. The formation of the soluble formazan was measured by light absorbance at 450 nm in a microplate reader. Cell proliferation was calculated by dividing the light absorbance in treated cells by that in control cells after correction for background absorbance.

Concentration dependent effects were analyzed by non-linear regression after log transformation of the concentration and graphs were fitted according to a sigmoid dose–response curve. A time-dependent effect was observed on GL-cell proliferation and results were reported after 72 h of incubation.

2.4. RNA isolation and synthesis of cDNA

Total RNA was isolated using the SV-total RNA isolation kit (Promega, Leiden, The Netherlands) according to the manufacturer’s protocol including a DNase treatment. The RNA was quantified spectrophotometrically at 260 nm (ND-1000, Nanodrop technologies) and stored at −70 °C.

First strand cDNA from 1 μg total RNA was synthesized with the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) containing both oligo (dT) and random hexamer primers in a final volume of 20 μL according the manufacturer’s recommendation. The cDNA was stored at −20 °C.

2.5. Real time quantitative PCR analysis

Gene-specific primers were developed, commercially synthesized (Eurogentec, Maastricht, the Netherlands) and tested for efficiency by using a dilution series of cDNA. The efficiency of the primers was between 95% and 105% and only one product was formed as assessed by melting curve analysis. 50 ng reverse transcribed RNA, 7.5 pmol of each gene-specific primer (Table 1) and IQ™ SybrGreen Supermix (Biorad) in a final 25 μL reaction volume was analyzed by quantitative PCR in a MyiQ single color real time PCR detection system (BioRad). Following an initial hot start for 3 min, 40 cycles were run with a denaturation step at 95 °C for 20 s, an annealing step at 63 °C for 30 s and an elongation step at 72 °C for 30 s.

2.6. Immunocytochemistry

For the immunocytochemical detection of ABC-transporters in the GL-1 and GL-40 cells, cytospin smears were prepared by placing 5 × 104 cells in culture medium into a cytology funnel with pre-attached filtercard (Biomedical Polymers Inc, Gardner (MA), USA) that was fixed with a funnel clip onto a polysine microscope slide (Menzel-Glaser, Braunschweig, Germany) and centrifuged at 650 rpm for 10 min in a cytospin centrifuge (Thermo Scientific™ Cytospin™ 4 CYtocentrifuge, Thermo Shandon Limited, Runcorn, UK). The freshly prepared cytospin preparations (1 × 104 cells/slide) were air dried, followed by a 3 min fixation step in acetone. After rehydration in phosphate-buffered saline (PBS) and incubation with 10% swine serum in PBS (20 min), endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol (Cell Marque, Rocklin, CA, USA) for 10 min. Slides were then incubated with mouse antibodies directed against human P-gp (Van der Heyden et al., 2011) (C494, Alexis Switzerland), MRP1 (Scheffer et al., 1994, Alexis Switzerland), and BCRP (Heyden et al., 2011) (C494, Alexis Switzerland).
et al., 2000) (MjIII-6, kind gift by dr Scheffer, the Netherlands) and BCRP (Maliepaard et al., 2001) (BPX-21, Abcam, UK) for 1 h at room temperature. Antibody binding was detected by polyvalent biotinylated serum, HRP-labeled streptavidin and diamobenzidine as a chromogen (Cell Marque, Rocklin, CA, USA). Following a 10-min wash in aqua dest, counterstaining was performed with hematoxyline solution according to Mayer (Sigma, St Louis, MO, USA) for 3 min, followed by a 2-min washing step in aqua dest.

2.7. Functional studies with fluorescent transporter substrates and selective inhibitors

Functional studies were performed using the typical fluorescent substrate-inhibitor combinations Rh123-PSC833 for assessing P-gp, CFDA-MK571 for MRP, and PhA-Ko143 for BCRP function (Allen et al., 2002; Dogan et al., 2004; Schleis et al., 2008). All experiments with GL-40 cells were performed with cells that had been incubated for one passage without doxorubicin. Before the GL-1 and GL-40 cells were used in experiments with fluorescent dyes, cell viability was assessed with trypan blue exclusion and cell numbers were counted in a hemocytometer. A total of 5 × 10^5 viable cells per sample were plated into 96-well plates. In the transport studies, culture medium was replaced by Hanks Balanced Salt Solution (HBSS) without Ca^2+, Mg^2+ and with 1000 mg/L glucose (Gibco, Grand Island, NY, USA). All chemicals were dissolved in DMSO as 1.000 mol/L) and cells were incubated for 30 min (37 °C, 5% CO2). Following this loading, cells were washed twice in PBS (Gibco, Grand Island, NY, USA) by centrifugation and the cell pellets were re-suspended in HBSS containing a concentration of 10,000 gated events per sample.

2.8. FACS data analysis

FACS data represent the geometric mean cell-associated fluorescence intensity and are reported in Relative Fluorescence Units (RFU). Changes in the cellular accumulation (Rh123, CF or PhA) by the inhibitors were presented as RFU. Efflux (Rh123, PhA) was calculated according to Eq. (1) and expressed as a percentage with one hundred percent efflux meaning that after two hours of incubation no fluorescence was measured within the cells and zero percent efflux meaning that all fluorescence was retained within the cells due to efflux inhibition.

\[
\text{Efflux} = \frac{(\text{Fl}_0 - \text{Fl}_b) - (\text{Fl}_{120} - \text{Fl}_b)}{(\text{Fl}_0 - \text{Fl}_b)} \times 100\%
\]  

(1)

\text{Fl}_0 is the Fluorescence intensity at t = 0 min, \text{Fl}_b is the Fluorescence intensity of the background, and \text{Fl}_{120} is the Fluorescence intensity of the samples at t = 120 min.

2.9. Glucocorticoid receptor assay

The presence of a functional glucocorticoid receptor was assessed in both GL-1 and GL-40 cells using a luciferase assay as previously described by Gracanin et al., 2012. In short, GL-cells (8 × 10^5 cells/well) were seeded in 24-well plates and transfected using per well 2 μL Lipofectamine 2000 (Invitrogen, Bleiswijk, the Netherlands), 800 ng of Mouse Mammary Tumor Virus (MMTV)-luciferase containing a glucocorticoid receptor-responsive reporter, and 0.3 ng of human β-actin-promoter renilla as an internal control. Following transfection the cells were left to recover for 24 h and then incubated with prednisolone (0.1 μmol/L) for 24 h. Firefly and renilla luciferase activities were subsequently quantified using a Dual-Luciferase Assay System (Promega, Leiden, the Netherlands) in a Centro LB 960 luminometer (Berthold Technologies, Vilvoorde, Belgium). The canine mammary cell line CNMm was used as a positive control.

2.10. Effect of prednisolone on ABC-transporter expression

The cells were incubated with a concentration range of prednisolone for 3, 6 and 24 h in cell culture medium (RPMI 1640). At the time of sampling, the cells were harvested by centrifugation, medium was discarded and the cell pellet was lysed in Promega lysis buffer. RNA isolation, cDNA synthesis and quantitative PCR analysis were performed as described above. Results are reported as Relative Expression that was calculated according to Eq. (2). Relative Expression = \(2^\Delta \Delta C_t\) 

\[
\Delta C_t = \text{Ct}_{\text{mean}} - \text{Ct}_{\text{sample}}
\]  

(2)

\text{Ct}_{\text{mean}} is the mean Ct value at t = 0 h for each ABC transporter for GL-1 or GL-40, and \text{Ct}_{\text{sample}} is the mean Ct value at t = 3, 6 or 24 h for each ABC transporter for GL-1 or GL-40.

2.11. Statistical analysis

All experiments were repeated independently for three times. Differences in accumulation or efflux of fluorescent dyes in the

### Table 1

<table>
<thead>
<tr>
<th>Canine gene</th>
<th>GenBank</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size</th>
<th>Ta (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcb1 (P-gp)</td>
<td>NM_001003215</td>
<td>CTAAGGCTTGGGCTTTCTTCT</td>
<td>GAGGGCGCTGGGAGATCTAC</td>
<td>80</td>
<td>57.5</td>
</tr>
<tr>
<td>Abcc1 (MRP)</td>
<td>NM_0010032971</td>
<td>GCTGCTGAGGGCAGGTGTT</td>
<td>CAGGTCACTCGGAGACCA</td>
<td>118</td>
<td>60.9</td>
</tr>
<tr>
<td>Abcg2 (BCRP)</td>
<td>NM_001048200</td>
<td>GGTATCCATAGCAACTCTCCT</td>
<td>GCCAAGGCGCACATACCAT</td>
<td>146</td>
<td>60.0</td>
</tr>
</tbody>
</table>
absence or presence of a specific transporter inhibitor were analyzed for statistic significance using the One-Way ANOVA with Dunnett multiple comparison post hoc test. The level of significance was set at $P < 0.05$ and significant differences are mentioned in the text. All statistical analyses were performed using Graph Pad Prism software (San Diego, California, USA).

### 3. Results

#### 3.1. GL-40. cells are resistant to doxorubicin and vincristine, but not prednisolone

The sub-cell line GL-40 was selected from GL-1 cells through intermittent incubation with gradually increasing concentrations of doxorubicin over a 6-month period and resulted with an increase in $IC_{50}$-doxorubicin from 17.2 nmol/L for GL-1 cells to 115 nmol/L for GL-40 cells. GL-40 cell showed resistance to vincristine with an $IC_{50}$-vincristine 0.54 nmol/L for GL-1 cells and 21.4 nmol/L for GL-40 cells (Fig. 1, Table 2). Prednisolone caused a mild (35%), but significant decrease in cell proliferation that was similar in both GL cell lines (Fig. 2).

#### 3.2. Immunocytochemistry of ABC-transporters P-gp, MRP1 and BCRP

P-gp expression was detected using the monoclonal antibody C494 and appeared more intense in GL-40 than GL-1 cells (Fig. 3). Using the monoclonal antibody M2III-6, MRP1 expression was demonstrated in both cell lines, but appeared more intense in GL-1 cells (Fig. 3). BCRP expression, using the monoclonal antibody BXP-21, was similar in both cell lines (Fig. 3).

#### 3.3. mRNA expression of the ABC-transporters P-gp, MRP1 and BCRP

P-gp mRNA expression was significantly lower in GL-1 cells ($Ct > 35$) than in GL-40 cells ($Ct = 22$), indicating an approximately

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**Table 2**

<table>
<thead>
<tr>
<th>Drug</th>
<th>PSC833</th>
<th>GL-1</th>
<th>GL-40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (nM)</td>
<td></td>
<td>17.2 (15.0–19.5)</td>
<td>115 (109–120)</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>13.3 (10.7–16.6)</td>
<td>15.8 (15.3–16.6)</td>
</tr>
<tr>
<td>Vincristine (nM)</td>
<td></td>
<td>0.54 (0.52–0.57)</td>
<td>21.4 (16.9–27.2)</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>0.32 (0.31–0.33)</td>
<td>0.31 (0.31–0.32)</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Inhibition of cell proliferation by doxorubicin (A and B) and vincristine (C and D) and the effect of PSC833 on drug sensitivity in GL-1 and GL-40 cells. Data are presented as mean ± SD of three independent experiments.

**Fig. 2.** Inhibition of cell proliferation by prednisolone in GL-1 and GL-40 cells. Data are presented as mean ± SD of three independent experiments.
7500 times higher expression in the latter (Fig. 4). MRP1 and BCRP expression were both detected (Ct values of 22 and 23 respectively), but similar in both GL-cell lines.

3.4. Functional studies for P-gp, MRP and BCRP

3.4.1. Rhodamine 123 as a probe dye for Pgp function
Rhodamine123 efflux from the GL-40 cells was approximately 4 times higher compared to the GL-1 cells (Fig. 4) and the P-gp-inhibitor PSC833 decreased Rh123 efflux in a concentration dependent fashion in the GL-40 cells (IC50 0.05 μmol/L), but not in the GL-1 cells (Fig. 5).

Rhodamine 123 accumulation was significantly lower in GL-40 compared to GL-1 cells and PSC833 caused a concentration dependent increase in Rh123 accumulation in the GL-40, but not in the GL-1 cells (Fig. 6). Only higher concentrations of MK571 (5 and 25 μmol/L) significantly increased Rh123 accumulation in the GL-40, but not the GL-1 cells (Fig. 6).

3.4.2. CFDA as a probe dye for MRP-transporters
Cellular accumulation of CF following incubation with CFDA (1 μmol/L) was higher in the GL-40 cells compared to the GL-1 cells and MK571 increased CF accumulation in a concentration dependent way in both GL cell lines (Fig. 7). The highest PSC833 concentration tested (1 μmol/L) resulted in a minor, but significant, increase in CF accumulation in the GL-1 cells at (Fig. 7).

3.4.3. Pheophorbide A as a probe dye for BCRP
PhA was tested at a concentration range from 0.1 to 1 μmol/L in combination with the BCRP inhibitor Ko143 at 1 μmol/L. PhA efflux

Fig. 3. Microscopic photographs of GL-1 (top row) and GL-40 (lower row) cells stained with May-Grünwald Giemsa (MGG) and immunocytochemical staining for P-gp, MRP1 and BCRP (columns).

Fig. 4. P-gp mRNA expression, measured by qPCR (left), and P-gp function, measured by rhodamine123 efflux (right), in the GL-1 and GL-40 cells, respectively. Data are presented as mean ± SD of three independent experiments.

Fig. 5. Efflux of the P-gp substrate rhodamine 123 by GL1 and GL-40 cells after 2 h of incubation in dye-free medium with increasing concentrations of PSC833. Data are presented as mean ± SD of three independent experiments.

Fig. 6. Efflux of the P-gp substrate rhodamine 123 by GL1 and GL-40 cells after 2 h of incubation in dye-free medium with increasing concentrations of PSC833. Data are presented as mean ± SD of three independent experiments.

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3.5. Doxorubicin and vincristine resistance in GL-40 cells is reversed by PSC833
PSC833 (1 μmol/L) restored GL-40’s sensitivity to the antiproliferative effects of doxorubicin and vincristine to levels comparable

Fig. 5. Efflux of the P-gp substrate rhodamine 123 by GL1 and GL-40 cells after 2 h of incubation in dye-free medium with increasing concentrations of PSC833. Data are presented as mean ± SD of three independent experiments.

(%) was similar in both GL-1 and GL-40 cells and decreased with increasing concentrations of PhA. Incubation with Ko143 had no effect on PhA efflux (Fig. 8). Cellular accumulation of PhA was comparable for both the GL-1 and GL-40 cell lines (Fig. 8), and Ko143 had no significant effect on the cellular PhA accumulation in either cell-line.

3.5. Doxorubicin and vincristine resistance in GL-40 cells is reversed by PSC833
PSC833 (1 μmol/L) restored GL-40’s sensitivity to the antiproliferative effects of doxorubicin and vincristine to levels comparable
with GL-1 cells (Table 2). PSC833 alone showed a mild, but equal, antiproliferative effect in both GL cell lines (Fig. 1).

3.6. GL-cells have a functional glucocorticoid-receptor, but prednisolone fails to induce P-gp, MRP1 or BCRP expression

Following transfection of CNMm, GL-1, and GL-40 cells with MMTV-luc, prednisolone (0.1 μM) caused a, respectively, 6-, 4-, and 2.6-fold increase in luciferase-activity confirming the presence of active glucocorticoid receptors in both cell-lines.

Short-term incubation (3, 6, 24 h) of both GL-1 and GL-40 cells with prednisolone did not significantly change the mRNA expression of P-gp, MRP1, and BCRP (Fig. 9). The low levels of P-gp mRNA expression in the GL-1 cells (Ct ≥ 35), resulted in a large variation in relative expression results.

4. Discussion

Selection for doxorubicin resistance by exposing the canine B-cell lymphoid leukemia cell line GL-1 (Nakaichi et al., 1996) to gradually increasing concentrations of doxorubicin led to the GL-40 sub-cell line that was six times more resistant to doxorubicin and showed cross-resistance to the structurally and mechanistically unrelated cytotoxic agent vincristine. The observed DR corresponded with an increased P-gp expression (mRNA and immunocytochemistry) and transport capacity for the P-gp model.
substrate Rh123. Both DR and Rh123-transport were completely reversed with the prototypical P-gp inhibitor PSC833. Therefore, it is concluded that P-gp causes multi-drug resistance in the GL-40 sub-cell line, which is in line with previous in vitro studies (Uozurmi et al., 2005; Matsuura et al., 2007). Furthermore these results indicate that in the dog, as in humans (Ambudkar et al., 1999), doxorubicin and vincristine are both P-gp substrates. Given the fact that P-gp expression is more prevalent in dogs with relapsed and DR cL (Bergman et al., 1996; Lee et al., 1996; Page et al., 2000), GL-40 cells represent a suitable in vitro model for studying DR in canine lymphoid neoplasia.

Induction of DR through incubation with increasing concentrations of a cytostatic agent, the method used in the current study, has a tendency to preferentially select for P-gp overexpression as the major DR-mechanism and might not necessarily reflect in vivo DR, which can be conveyed through both other efflux-transporters, as well as other mechanisms (Calcagno and Ambudkar, 2010). For example, besides increased P-gp expression, changes in cellular survival signaling cascades, resistance to apoptosis and upregulation of antioxidant defense enzymes (McCubrey et al., 2006; Tome et al., 2012) have been shown to result in DR and these mechanisms might also have contributed to GL-40’s DR phenotype.

Other ABC-transporters implicated in DR to cytostatic drugs in humans include MRP1 and BCRP. The canine orthologs of both these transporters have been evaluated in transfected cell lines and have associated MRP1 with resistance to vincristine, but not doxorubicin (Ma et al., 2002) and BCRP with resistance to doxorubicin (Honscha et al., 2009). In the GL-40 cells the level of MRP1 mRNA expression was similar to that of the parental GL-1 cells, although the less intense immunoreactivity and the higher CF retention in the GL-40 cells suggest a reduced MRP1 protein-expression and function. BCRP expression (mRNA, immunocytochemistry) and function (PhA transport) seemed low and equal for both GL cell-lines. Therefore, it can be assumed that neither MRP1, nor BCRP are a cause for GL-40’s observed DR.

Prednisolone had a mild and equal antiproliferative effect on both GL-1 and GL-40 cells. The absence of prednisolone resistance in the GL-40 cells is not consistent with the assumption that prednisolone is a P-gp substrate and stands in contrast with human data (Crowe and Tan, 2012) and a canine in vivo study (Van der Heyden et al., 2012). The absence of a functional glucocorticoid

Fig. 9. The relative mRNA expression of P-gp, MRP1 and BCRP in the GL-1 and GL-40 cells following incubation with prednisolone (μmol/L) for 0, 3, 6, and 24 h. Data are presented as mean ± SD of three independent experiments.
receptor would explain this finding, but is rejected since a luciferase assay demonstrated activation of glucocorticoid receptor response elements following exposure to prednisolone in both GL cell lines. Alternative mechanisms for this unexpected glucocorticoid resistance would include down-stream effects like resistance to glucocorticoid-induced apoptosis (Schlossmacher et al., 2011). This observation, as well as the cause for the lower luciferase-activity activity in the GL-40 cells, requires further investigation. Furthermore, prednisolone failed to induce expression of P-gp, MRP1 and BCRP mRNA in both GL-1 and GL-40 cells. Based on these data, prednisolone appears neither a substrate for, nor an inducer of P-gp in canine lymphoid cells, which makes the assumption that prednisolone treatment leads to DR through induction of P-gp overexpression unlikely.


