Antitumour effect and modulation of expression of the ABCB1 gene by perifosine in canine lymphoid tumour cell lines

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ABSTRACT

Acquisition of multidrug resistance (MDR) is a common cause of treatment failure during chemothera-
py for dogs with lymphoma (lymphosarcoma). Overexpression of P-glycoprotein (P-gp), encoded by the
ABCB1 gene, is associated with MDR. Perifosine, an Akt inhibitor, downregulates the expression of P-gp.
In this study, the antitumour effect of perifosine and its ability to modulate ABCB1 expression were ex-
amined in four canine lymphoid tumour cell lines (GL-1, CLBL-1, UL-1 and Ema). GL-1 and CLBL-1 were
inherently negative for P-gp, while UL-1 and Ema were inherently positive for P-gp. GL-1 and UL-1 were
sensitive to perifosine, whereas CLBL-1 and Ema were resistant. The amount of ABCB1 mRNA signifi-
cantly decreased after treatment with perifosine in UL-1, associated with activation of the c-Jun NH2-
terminal kinase (JNK) pathway, but such an effect was not observed in Ema. In UL-1, perifosine decreased
the efflux of rhodamine 123 dye and reduced the 50% inhibitory concentration of vincristine, but such
effects were not observed in Ema. Perifosine had an antitumour effect in 2/4 canine lymphoid tumour
cell lines. In 1/4 cell lines, perifosine downregulated ABCB1 gene expression through activation of the JNK
pathway and increased sensitivity to vincristine.

Introduction

Lymphoma (lymphosarcoma) is the most common haematological neoplasm in dogs (Dobson et al., 2001). In many
cases, chemotherapy for lymphoma initially is effective; however, lymphoma cells frequently acquire a multidrug resistance (MDR)
phenotype during chemotherapy and treatment may fail (Marconato, 2011). Therefore, it is important to elucidate the mechanisms that
confer an MDR phenotype on tumour cells and to determine effective therapies for tumour cells with MDR.

Various molecules induce a MDR phenotype in tumour cells from human beings (O’Connor, 2007) and companion animals (Bergman,
2003). Acquisition of the MDR phenotype is thought to be multifactorial and the overexpression of efflux pumps, such as the ATP-
binding cassette (ABC) transporter family, is one of the important factors in MDR (Bergman, 2003). Among ABC transporters, the efflux
pumps encoded by the ABCB1, ABCC1 and ABCG2 genes are frequently associated with the acquisition of MDR (Modok et al., 2006).
In addition to ABC transporters, overexpression of lung resistance-related protein (LRP) is also associated with acquisition of MDR
(Gromicho et al., 2011).

Overexpression of such transporters reduces the intracellular concentration of chemotherapeutic agents, which can be substrates of
these transporters and are effluxed. In veterinary medicine, although little is known about the association of LRP with MDR in
tumour cells, the expression of P-glycoprotein, encoded by ABCB1, is associated with the MDR phenotype in dogs (Moore et al., 1995;
Bergman et al., 1996; Lee et al., 1996). However, the mechanisms that induce P-gp overexpression are not fully understood and ef-
fective therapies to overcome the MDR phenotype acquired as a result of P-gp overexpression have not yet been established.

Perifosine is an anticancer drug that inhibits the phosphoinositide 3-kinase (PI3K)/Akt pathway. It has been used in clinical trials as a
treatment for human patients with colorectal cancer and multiple myeloma (Srivastava and Cho, 2013). Perifosine inhibits activation of
the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways and activates the c-Jun NH2-
terminal kinase (JNK) pathway. Perifosine downregulates the expression of P-gp through activation of the JNK pathway (Chiarini et al., 2008).

Recently, we reported that ABCB1 and LRP are upregulated by the MAPK/ERK pathway in canine lymphoma cell lines (Tomiyasu et al.,
2013a). On the basis of this previous study, we hypothesised that modulation of intracellular signalling by treatment with perifosine
could decrease expression of ABCB1 and LRP. There have been no reports of the antitumour effects of perifosine or its effects on mod-
ulation of the expression of drug efflux pumps in canine tumour cells. The aim of the present study was to determine whether perifosine
exerts an antitumour effect or affects the expression of ABCB1 or LRP in canine lymphoid tumour cell lines.
**Materials and methods**

**Cell lines**

Four canine lymphoid tumour cell lines were used in this study: GL-1, a canine B cell leukaemia cell line (Rutgen et al., 2010); UL-1, a canine T cell lymphoma cell line (Yamazaki et al., 2008); and Ema, a canine T cell lymphoma cell line (Hirakoa et al., 2009). GL-1 and CLBL-1 are inherently negative for ABCB1 and P-gp expression, whereas UL-1 and Ema inherently express ABCB1 and P-gp (Tomiyasu et al., 2013b). All cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum and cultured at 37°C in a humidified atmosphere of 5% CO₂.

**Drug sensitivity assay**

The 50% inhibitory concentration (IC₅₀) values for perifosine were determined in the four cell lines. Cells were cultured at 2 × 10⁴ cells/ml in 96-well plates in media containing 0.1–100 μM perifosine (Selleck, 51137) for 48 h. The viability of the cells after cultivation was measured using Cell Counting Kit-8 (Dojindo). Absorbance was measured using a Model 680 Microplate Reader (Bio-Rad Laboratories) and IC₅₀ values were determined. In addition, IC₅₀ values for vincristine sulphate (Oncovin, Nippon Kayaku) following simultaneous incubation of cells with perifosine were examined in UL-1 and Ema. Cell viabilities were measured after simultaneous treatment with perifosine and various concentrations of vincristine sulphate (1, 5, 10, 20, 30, 50, 100 and 500 ng/ml in UL-1; 1, 5, 10, 50, 100 and 500 ng/ml in Ema) for 48 h. In this assay, Ema cells were treated with 20 μM perifosine because this concentration was near the maximum trough level of perifosine obtained in a clinical trial in human beings (Cru hdf, 2002). However, this concentration of perifosine showed an apparent cytotoxic effect in UL-1; therefore, a lower concentration of perifosine (5 μM) was used for this cell line. The cell viabilities relative to cells treated with perifosine alone were measured as described above. To examine the IC₅₀ values for vincristine in UL-1 and Ema without treatment with perifosine, cells were incubated with the same concentrations of vincristine sulphate as described above for 48 h and cell viability was determined.

**Western blot analysis for PI3K/Akt, MAPK/ERK and JNK pathways**

Western blot analysis was used to examine the activation statuses of the PI3K/Akt, MAPK/ERK and JNK pathways in cell lines after treatment with 20 μM perifosine. In these standard curves, the relative quantities of cDNA for ABCB1, LRP and TBP included in 50 ng of this cDNA sample were defined as 1 × 10⁵. These standard curves were examined in triplicate. The parameters of the standard curves are shown in Appendix A (see Supplementary material).

**Drug sensitivity assay**

Inhibition of JNK or PI3K/Akt pathways

To determine the association between ABCB1 and LRP gene expression and signalling pathway activation in the UL-1 and Ema cell lines, SP600125 (Wako, 197-16591) and LY294002 (Cell Signaling Technology, 9901) were used to inhibit the JNK and PI3K/Akt pathways, respectively, as previously described (Chang et al., 2011; Zhu et al., 2012). For inhibition of JNK pathway, cells were treated with 50 μM SP600125 for 40 min before incubation with 20 μM perifosine for 8 or 12 h for extraction of total protein or total RNA, respectively. The amounts of ABCB1 and LRP mRNAs, and the amounts of phospho-SAPK/JNK, SAPK/JNK, phospho-c-Jun and c-Jun, were compared among untreated cells, cells treated with perifosine alone and cells treated with both perifosine and SP600125 using RT-PCR and Western blot analysis, as described above. These quantities were performed in triplicate.

**Rhodamine 123 efflux test**

UL-1 or Ema cells (1 × 10⁴), with or without pretreatment with 20 μM perifosine for 12 h, were incubated with 200 ng/ml rhodamine 123 (Rh) (Sigma-Aldrich, R8004) in RPMI 1640 medium at 37°C for 20 min. After washing in phosphate buffered saline (PBS), the cells were incubated in Rh-free medium at 37°C for 60 min, either with or without 2 μM of cyclosporine (Cs) (Cyclosporin A, Wako, 035-16301). Cells that had not been exposed to either Rh or Cs were used as negative controls. Following incubation, the cells were washed with PBS and subjected to flow cytometric analysis (FACS Calibur; Becton Dickinson). The Rh efflux index (REI) was calculated as (mean

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**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Oligonucleotide primer</th>
<th>Amplicon size (base pairs)</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>F</td>
<td>5'-ACTCCGCGACAGCAATTTGTG-3' (2734–2753)</td>
<td>95</td>
<td>NM01003215</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-AATGACACCCGCCAGATTGTG-3' (2809–2828)</td>
<td>144</td>
<td>XM36910</td>
</tr>
<tr>
<td>LRP</td>
<td>F</td>
<td>5'-CAAGACCCCTCTGTGGTACG-3' (1358–1377)</td>
<td>96</td>
<td>XM849432</td>
</tr>
<tr>
<td>TBP</td>
<td>F</td>
<td>5'-CTATTGTTGTTGCTGTCAGGG-3' (1345–1360)</td>
<td>144</td>
<td>XM36910</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CTCCTGACATGCTTCTTT-3' (31–50)</td>
<td>96</td>
<td>XM849432</td>
</tr>
</tbody>
</table>

F, forward (sense); R, reverse (antisense).
fluorescence intensities of the sample incubated with Rh and Cs – those of the negative control)/(those of the sample incubated with Rh – those of the negative control). The Rh efflux test was performed five times.

**Statistical analysis**

The nonparametric Mann–Whitney U test was used to compare the relative levels of mRNA expression of ABCB1 and LRP, and REIs. Repeated measures two-way analysis of variance (ANOVA) and Bonferroni post tests were used to compare cell viabilities after incubation with vincristine between cells with and without perifosine treatment. All statistical tests were two sided and performed using Prism 5 (GraphPad). For all tests, \( P < 0.05 \) was used as the level of significance.

**Results**

**Cytotoxic effect and modulation of intracellular signalling by perifosine**

The IC\(_{50}\) values for perifosine in GL-1, CLBL-1, UL-1 and Ema were 9.91, 33.0, 7.01 and 58.7 \( \mu \text{M} \), respectively (Fig. 1). Western blot analysis showed increased amounts of phospho-SAPK/JNK, phospho-c-Jun, and c-Jun following treatment with perifosine in GL-1 and UL-1, although these effects were not observed in CLBL-1 and Ema (Fig. 2). In the MAPK/ERK pathway, a decrease in the amount of phospho-ERK1/2 was observed in GL-1, UL-1 and Ema following treatment with perifosine, but this effect was not observed in...
In addition, decreases in the amounts of phospho-Akt (Ser473) and phospho-Akt (Thr308) were observed in GL-1 and UL-1; however, these effects were not observed in CLBL-1 and Ema.

Changes in quantities of ABCB1 and LRP mRNAs by perifosine

Treatment with perifosine significantly decreased the amounts of ABCB1 (0.538-fold) and LRP (0.823-fold) mRNAs in UL-1 (Figs. 3a and b); however, the extent of the decrease of LRP gene expression in this cell line was within the intra-assay error (≥0.81-fold difference). In contrast, the amounts of both ABCB1 and LRP mRNAs did not change significantly after treatment with perifosine in Ema (Fig. 3c and d).

Effects of inhibition of JNK or PI3K/AKT pathways

In UL-1, the increase in phospho-SAPK/JNK, phospho-c-Jun and c-Jun following treatment with perifosine was inhibited by treatment with SP600125 prior to exposure to perifosine (Fig. 4a). The amount of ABCB1 mRNA was significantly increased when UL-1 cells were incubated with both perifosine and SP600125 (0.698-fold) compared to perifosine alone (Fig. 4b). However, the amounts of LRP mRNA were not significantly different between UL-1 cells treated with both perifosine and SP600125, and those treated with perifosine alone (Fig. 4c).

When PI3K/Akt pathway was inhibited using LY294002, decreased amounts of phospho-Akt (Ser473) and phospho-Akt (Thr308) were observed in both UL-1 and Ema (Fig. 5a). However, the amounts of ABCB1 or LRP mRNAs were not significantly different between the cells treated with LY294002 and control cells in both UL-1 and Ema (Figs. 5b–e).

Changes in drug efflux function by treatment with perifosine

An Rh efflux test was performed to determine the effects of perifosine on drug efflux function of ABC transporters. There were no differences in intracellular fluorescence intensities of Rh in cells incubated with Rh and Cs between control cells (Fig. 6a) and cells treated with perifosine (Fig. 6b). Fluorescence intensities in cells incubated with Rh alone were increased in cells treated with perifosine compared with control cells in UL-1, but not Ema (Figs. 6c and d). REI values were significantly decreased in UL-1 by treatment with perifosine compared with control cells (4.65 vs. 7.29, respectively; Fig. 6e), but there was no significant difference between cells treated with perifosine and control cells in Ema (8.36 vs. 9.73, respectively; Fig. 6f).

Effect of perifosine on sensitivity of canine lymphoma cell lines to vincristine

Cell viabilities were examined after treatment with vincristine, with or without simultaneous exposure to perifosine, to determine whether perifosine affected the sensitivity of cells to vincristine. In UL-1 cells treated with 20 or 30 ng/mL vincristine, cell viabilities were significantly lower in cells exposed to perifosine than in cells not exposed to perifosine (Fig. 7a). The IC50 values for vincristine were 15.6 and 29.6 ng/mL, respectively, in cells with and without treatment with perifosine. In Ema, there was no significant difference in cell viabilities between control cells and cells treated with perifosine at any concentration of vincristine (Fig. 7b). The IC50 values for vincristine were 77.8 or 81.9 ng/mL, respectively, in cells with or without treatment with perifosine.

Discussion

This study showed that IC50 values for perifosine in GL-1, CLBL-1, UL-1 and Ema were 9.91, 33.0, 7.01 and 58.7 μM, respectively. In clinical trials in human medicine, the trough levels of perifosine obtained with a dosage of 200 mg/day perifosine were 2.6–8.2 μg/mL (5.6–17.8 μM; Crul et al., 2002). In the present study, the IC50 values for perifosine in GL-1 and UL-1 were lower than or compatible with the trough levels of perifosine in human beings.
Therefore, 2/4 cell lines used in the present study appeared to be sensitive to perifosine.

Treatment with perifosine inhibited the PI3K/Akt and MAPK/ERK pathways, and activated the JNK pathway, in agreement with the results of a previous study (Chiarini et al., 2008). The present study showed that modulation of these intracellular signalling pathways by perifosine differed among four canine lymphoma cell lines. The PI3K/Akt, MAPK/ERK and JNK pathways were modulated in GL-1 and UL-1, which were sensitive to perifosine. In two perifosine-resistant cell lines, none of these pathways were modulated in CLBL-1, whereas only the MAPK/ERK pathway was modulated in Ema. Perifosine exerts an antitumour effect mainly owing to its ability to inhibit the PI3K/Akt pathway (Schmidt-Hieber et al., 2012). Similarly, inhibition of the PI3K/Akt pathway may mediate the antitumour effect of perifosine in canine lymphoid tumour cells.

Of the four cell lines used in the present study, GL-1 and CLBL-1 were B cell lines, whereas UL-1 and Ema were T cell lines. However, the immunophenotype was not related to differences in the effects of perifosine; the PI3K/Akt, MAPK/ERK and JNK pathways were modulated by perifosine in GL-1 and UL-1, none of these pathways was modulated in CLBL-1 and only the MAPK/ERK pathway was modulated in Ema. Perifosine modulates the PI3K/Akt, MAPK/ERK and JNK pathways by targeting Akt, RasGRP/Raf (upstream of ERK) and ASK1 (upstream of JNK), respectively (van Blitterswijk and Verheij, 2013). Further studies are needed to identify mutations or other factors that determine the effects of perifosine on intracellular signalling in each of these pathways in canine lymphoma cell lines.

The ability of perifosine to modulate the expression of ABCB1 and LRP, as well as the function of drug efflux pumps, was examined in UL-1 and Ema, which are inherently positive for ABCB1 and express P-gp (Tomiyasu et al., 2013b). Exposure to perifosine induced a significant decrease in the amount of ABCB1 mRNA in UL-1. Downregulation of ABCB1 gene expression in this cell line by perifosine was significantly inhibited by SP600125, a JNK inhibitor, although treatment with LY294002, a PI3K inhibitor, had no effect on ABCB1 mRNA levels. These observations suggest that perifosine downregulates ABCB1 gene expression at least in part through activation of the JNK pathway, whereas inhibition of PI3K/Akt pathway

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**Fig. 4.** Activation of the JNK pathway by perifosine and its inhibition by SP600125 (a). Fold-changes of relative quantities of ABCB1 (b) and LRP (c) mRNA induced by treatment with perifosine alone or perifosine plus SP600125 in UL-1. *P* < 0.05 vs. control. †P < 0.05 vs. cells treated with perifosine.
might not be involved in downregulation of expression of this gene. Downregulation of P-gp expression through activation of the JNK pathway by perifosine has also been reported in human studies (Chiarini et al., 2008). Our previous study showed that activation of the MAPK/ERK pathway upregulates expression of ABCB1 and LRP in UL-1 and Ema (Tomiyasu et al., 2013a). However, treatment with perifosine did not significantly alter the amount of ABCB1 mRNA in Ema, despite inhibiting the MAPK/ERK pathway. Inhibition of the JNK pathway might be insufficient, whereas activation of the JNK pathway might be important, for downregulation of ABCB1 by perifosine. Differential activation of the JNK pathway might be responsible for differences in modulation of ABCB1 expression by perifosine in UL-1 and Ema.

Treatment with perifosine also affected the drug efflux function of UL-1 cells, in which perifosine suppressed ABCB1 expression. Efflux of Rh was decreased in UL-1 by treatment with perifosine, indicating that the drug efflux function of ABC transporters is suppressed by perifosine. Although perifosine significantly decreased expression of LRP, the extent of the decrease did not exceed the level of intra-assay error in the real-time PCR and the effect of the decrease of LRP gene expression on drug efflux function was unclear in UL-1. In addition, no significant change in LRP gene expression level was observed in Ema. These results indicate that mechanisms other than changes in activation status of the MAPK/ERK, JNK and PI3K/Akt pathways might be important in the regulation of LRP gene expression. Further studies are needed to clarify the mechanisms of regulation of LRP gene expression.

The IC50 value for vincristine was lower (15.6 ng/mL) in UL-1 as a result of exposure to perifosine compared to untreated cells (29.6 ng/mL), whereas there was no significant difference in IC50 values for vincristine between Ema cells exposed to perifosine (77.8 ng/mL) or not exposed (81.9 ng/mL). UL-1 and Ema were resistant to vincristine alone, whereas UL-1 became sensitive to vincristine in the presence of perifosine. There are no studies on the
pharmacokinetics of vincristine in dogs, but values for \( C_{\text{max}} \) of vincristine were 21.9 and 28.6 ng/mL in two human patients when administered at a dosage of 0.05 mg/kg (Skolnik et al., 2006). A dosage of 0.7 mg/m\(^2\) vincristine is recommended in representative chemotherapeutic protocols for dogs with lymphoma (Keller et al., 1993; Moore et al., 2001; Garrett et al., 2002).

Conclusions

In some canine lymphoma cell lines, perifosine downregulated \( ABCB1 \) expression through activation of the JNK pathway and increased sensitivity to vincristine. Combination chemotherapy of perifosine and anticancer drugs that are substrates for P-gp should be considered as a treatment for canine lymphoma.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A: Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2014.04.002.

References
