Effects of toceranib phosphate (Palladia) monotherapy on multidrug resistant lymphoma in dogs

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ABSTRACT. We examined whether multidrug resistant (MDR) canine lymphoma increases gene expression for platelet-derived growth factor receptor α (PDGFRα), vascular endothelial growth factor receptor 2 (VEGFR2), and c-KIT, and whether toceranib phosphate (TOC) has potential as a treatment for MDR canine lymphoma. The clinical data showed that PDGFRα expression was higher in canine subjects with MDR T-cell lymphoma than in those with untreated T-cell lymphoma, and that c-KIT expression was greater in subjects with T-cell lymphoma than in those with B-cell lymphoma. TOC monotherapy was well tolerated without serious adverse effects, and two of the five subjects that received TOC exhibited partial responses. The data presented here could contribute to the assessment of TOC-based therapy for dogs with MDR or T-cell lymphoma.

KEY WORDS: dog, lymphoma, multidrug resistance, toceranib phosphate

Although multidrug therapy based on doxorubicin (DOX) is highly effective against canine lymphoma, it leads to multidrug resistant (MDR) lymphoma and, ultimately, to treatment failure [17]. It has been reported that this resistance is facilitated by the activation of P-glycoprotein (P-gp), other drug transporters, or a combination of both [18]. Angiogenesis is required for the survival of hematologic cancers [20] and the expression patterns of angiogenic growth factors, including platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR), correlate with clinical stage and historical grade in canine lymphoma [2, 15]. Additionally, overexpression of PDGFR and VEGFR promotes resistance to several anti-cancer drugs [1, 14]. Therefore, determining the relationship between multidrug resistance and the expression of PDGFR and VEGFR in canine lymphoma may provide medically valuable information for its treatment.

Toceranib phosphate (TOC) is a tyrosine kinase inhibitor (TKI) that targets PDGFR, VEGFR and KIT. It is approved for the treatment of canine mast cell tumors, and some groups have reported its efficacy in dogs with several types of carcinomas [3, 7, 13]. Other groups have demonstrated that TOC has high bioavailability and is usually well tolerated [16]. Masitinib, another TKI that targets PDGFR, VEGFR and KIT, also inhibits the growth of canine lymphoma cells in vitro [6, 19]. Moreover, the same groups have reported that KIT is expressed at high levels in dogs with high-grade T-cell lymphomas, and that masitinib shows antitumor effects in these dogs [5, 6]. However, whether TOC can be used to treat canine lymphoma is unknown. Evaluating its effects may ultimately improve the treatment of MDR canine lymphoma.

In this study, our aim was to determine the effect of chemoresistance on the expression patterns of PDGFRα, VEGFR2 and...
than the upper limit of the reference range, or albumin 0.75 times lower than the lower limit of the reference range. If subjects were undergoing therapy, adverse events were recorded and graded every two weeks in accordance with the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events.

Animal Care and Experimentation at Kagoshima University was performed according to the Institutional Guidelines for Animal Experiments and in compliance with the Ethics Committee of Animal Care and Experimentation at Kagoshima University. Client consent for the TOC-based clinical trial was obtained from client owners of five out of the 16 subjects with lymphoma; the expression of the target genes, ATP binding cassette B1 (ABCB1), ATP binding cassette G2 (ABCG2), PDGFRα, VEGFR2, and c-KIT, in lymphoma cells and tissue samples, were evaluated using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) after isolation of total RNA. Details are included in the Supplementary Methods, including Tables and Figures.

Samples were collected from 16 dogs with lymphoma during their first visits to a veterinary teaching hospital at Kagoshima University between 2012 and 2016. All subjects were treated with a standard multidrug protocol (L-asparaginase, cyclophosphamide, DOX, vincristine, and prednisone) as a first-line therapy. In 12 out of 16 subjects, samples were collected for a second time, from a relapsed tumor after the first-line therapy failed. The following were excluded from sampling: (1) subjects affected with MDR lymphoma with high levels of expression of PDGFRα, VEGFR2 and c-KIT, and that TOC had potential as an effective canine anticancer therapy.

Canine lymphoma cell lines (CL-1 and GL-1) [8, 10] and DOX-resistant lymphoma cells were used in this study. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, U.S.A.), supplemented with 10% heat-inactivated fetal bovine serum (Cosmo Bio, Tokyo, Japan) and 1% L-glutamine (BioWhittaker, Walkersville, MD, U.S.A.) with 5% CO2 at 37°C. DOX-resistant cells were generated from the CL-1 and GL-1 lines in accordance with a previously reported procedure [18], the details of which are given in the Supplementary Methods, including Tables and Figures.

Table 1. Summary of clinical information for the 16 canine subjects with lymphoma

| No. | Age (year) | Sex a | Breed b | Location | Type c | Stage d | Protocol e | Response f | PFI h (days) | Sampling b |
|-----|------------|-------|---------|----------|--------|--------|-----------|------------|--------------|-------------|
| 1   | 10         | M     | MD      | Multicentric | B      | 2a     | CHOP      | CR         | 336          | Twice       |
| 2   | 12         | F     | BG      | Multicentric | B      | 3a     | CHOP      | CR         | 252          | Twice       |
| 3   | 9          | M     | WC      | Multicentric | T      | 3a     | LCHOP     | PR         | 105          | Twice       |
| 4   | 14         | M     | MD      | Multicentric | B      | 4b     | LCHOP     | CR         | 189          | Once        |
| 5   | 7          | F     | MS      | GI tract | T      | /      | LCHOP     | SD         | 42           | Once        |
| 6   | 10         | M     | GS      | Multicentric | B      | 3a     | LCHOP     | CR         | 168          | Twice       |
| 7   | 8          | F     | FB      | Multicentric | B      | 4b     | CHOP      | CR         | 420          | Twice       |
| 8   | 13         | F     | MD      | Cutaneous | T      | /      | LCHOP     | PR         | 126          | Twice       |
| 9   | 9          | F     | LR      | Multicentric | B      | 3b     | LCNOP     | CR         | 210          | Twice       |
| 10  | 14         | F     | SS      | Multicentric | B      | 4b     | LCNOP     | PR         | 147          | Once        |
| 11  | 9          | M     | WC      | Multicentric | B      | 3a     | CHOP      | CR         | 294          | Twice       |
| 12  | 10         | F     | Pug     | Multicentric | B      | 3b     | LCHOP     | CR         | 378          | Twice       |
| 13  | 8          | M     | MD      | Mediastinal | T      | /      | LCHOP     | PD         |              | Once        |
| 14  | 13         | F     | MS      | Multicentric | B      | 2a     | CHOP      | CR         | 315          | Twice       |
| 15  | 12         | F     | BH      | Multicentric | B      | 3a     | LCHOP     | CR         | 336          | Twice       |
| 16  | 10         | M     | Mix     | GI tract | T      | /      | LCHOP     | SD         | 63           | Twice       |

a) F: Female, M: Male. b) BG: Beagle, BH: Basset Hound, FB: French Bulldog, GS: German Shepherd Dog, LR: Labrador Retriever, MD: Miniature Dachshund, MS: Miniature Schnauzer, SS: Shetland Sheepdog, WC: Welsh Corgi. c) B: B-cell Lymphoma, T: T-cell Lymphoma. d) Clinical staging of multicentric lymphoma at first visit (extranodal lymphoma was not shown). e) L: L-asparaginase, C: Cyclophosphamide, H: Doxorubicin, N: Mitoxantrone, O: Vincristine, P: Prednisone. f) CR: Complete Response, PR: Partial Response, SD: Stable Disease, PD: Progressive Disease. g) PFI: Progression-free interval. h) Once: Sample was collected before treatment, Twice: Samples were collected before and after treatment.

c-KIT in canine lymphoma, and how TOC affects dogs with MDR lymphoma. We hypothesized that MDR lymphoma would cause high levels of expression of PDGFRα, VEGFR2 and c-KIT, and that TOC had potential as an effective canine anticancer therapy.
developed signs of a protein-losing syndrome (UP/C >2, albumin <0.75 times the lower limit of the reference range), therapy was interrupted until values had returned within acceptable limits. If this was observed for a second time, treatment was permanently discontinued. If hemolytic anemia (hemoglobin (Hb) <10 g/µl and bilirubin >1.5 times the upper limit of normal) or anemia without regeneration (Hb <10 g/µl and reticulocytes <80,000/µl) occurred, treatment was discontinued. In cases of hepatic toxicity (alanine aminotransferase, aspartate aminotransferase, or total bile acid >3 times the upper limit of normal) or neutropenia (<2,000 cells/µl), treatment was interrupted until values normalized, then continued at the same dosage used previously. If these events occurred for a second or third time, treatment was interrupted until they were resolved, then resumed at a lower dose of 2.0–2.4 mg/kg. If severe adverse reactions occurred even at this low dose, treatment was permanently discontinued. If one of the previously mentioned adverse reactions, severe diarrhea or severe vomiting, persisted after dose reduction, treatment was permanently discontinued.

Clinical response to TOC was assessed every three weeks in accordance with Response Evaluation Criteria in Solid Tumors (RECIST) [11]: complete response (CR) (disappearance of all clinical signs of disease); partial response (PR) (>50% or <100% decrease); stable disease (SD) (≤50% decrease or <20% increase); and progressive disease (PD) (≥20% increase and/or development of new lesions or metastases). The progression-free interval (PFI) included CR, PR and SD through to at least day 21. Survival time (ST) was calculated from the date of the first treatment through to the date of death. The data for subjects without follow up data prior to death or that died of another cause, were excluded from the ST data.

Statistical analyses were performed using standard software (IBM SPSS Statistics, SPSS Inc., Chicago, IL, U.S.A.). Data were analyzed using Dunnett’s test or one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. Quantitative values of the data from three separate experiments were expressed as mean ± standard deviation (SD), and P-values less than 0.05 were considered significant.

Ninety and 180 days after culture in medium that included low doses of DOX, CL-1 and GL-1 cells demonstrated time-dependent decreases in sensitivity to DOX that were significantly greater than the decreases seen in control cells (Fig. S1). The IC_{50} values of CL-1 and GL-1 control cells were 5.8 and 1.40 nM respectively, and IC_{50} values of CL-1 and GL-1 cells 180 days after culture were 98.2 and 128.2 nM, respectively. At 180 days after culture, expression of P-gp was upregulated in the GL-1 and CL-1 cells, and the relative intensities of the immunoreactive bands in GL-1 cells were significantly greater than those of the controls (Fig. S2). The relative expression of ABCB1 and ABCG2 mRNA in the CL-1 and GL-1 cells increased time-dependently while the cells were being cultured, and expression levels were significantly higher in these cells than in the controls 180 days after culture (Fig. S3). The data demonstrates that the cells developed DOX-resistance because of increases in activity of drug transporters. Moreover, the relative expression of PDGFRα, VEGFR2, and c-KIT also increased time-dependently and simultaneously during culture. At 180 days post-culture, expression levels of these genes were significantly different to levels in the control cells. The data suggests that expression of PDGFRα, VEGFR2, and c-KIT increased after the cells developed DOX resistance.

After the failure of standard multidrug therapy, the relative expression levels of ABCB1 and ABCG2 were significantly higher in MDR lymphomas than in untreated lymphomas (Fig. 1): this demonstrated that chemoresistance developed due to an increase in drug transporter expression. Moreover, the relative expression of PDGFRα was significantly higher in MDR T-cell lymphomas than in untreated T-cell lymphomas. Expression of c-KIT did not differ significantly between MDR and untreated lymphomas, although was significantly higher in T-cell lymphomas than in B-cell lymphomas (Fig. 1). These results suggest that the pathogenic mechanism of chemoresistance correlates with the expression of PDGFRα in T-cell lymphoma, and that TOC might show antitumor effects on MDR T-cell lymphoma in dogs with elevated PDGFRα and c-KIT expression.

Five subjects received TOC for MDR lymphoma (Table 2). The tumor responses in two of these subjects (Numbers 8 and 16) were classified as PRs for 105 and 84 days, respectively. The response in one subject (Number 2) was classified as a SD for 42 days, but the responses in the other subjects (Numbers 6 and 12) were classified as PDs. The two subjects with tumor responses classified as PRs had diarrhea and neutropenia one month after the administration of TOC; however, these adverse events were mild and limited, and they did not result in discontinuation. Of the five subjects that received TOC, expression levels of the targeted genes were highest in the group with responses classified as PRs: expression levels of PDGFRα and c-KIT were highest in Number 8, and the expression level of VEGFR2 was the highest in Number 16 (Fig. 1). After TOC monotherapy failed, all subjects were retired from the clinical trial. For four of the five subjects, the owners decided to continue administering the medication beyond the end of the clinical trial. Three (Numbers 2, 6 and 8) of the four subjects were treated with lomustine (CCNU, 50–70 mg/m², orally, every three weeks), and another subject (Number 16) received nimustine (ACNU, 30–35 mg/m², intravenously, every three weeks) as a rescue therapy. Number 12 received supportive care without additional chemotherapy after the clinical trial.

In these studies, we demonstrated that expression of PDGFRα was significantly higher in subjects with MDR T-cell lymphomas than in those with untreated T-cell lymphomas, and c-KIT expression was greater in subjects with T-cell lymphomas than in those with B-cell lymphoma. Overexpression of PDG and PDGFR is a major player in oncogenesis and drug resistance [14]. Moreover, one study suggested that c-KIT might be expressed on canine lymphomas, depending on their origin or histological grade, and is related to clinical outcomes [5]. Therefore, we suggested that there was a mismatch in the c-KIT expression patterns between in vitro and clinical samples due to the difference in origin, histological grade or clinical stage. On the basis of these findings, we believe that multidrug resistance and its development in T-cell lymphoma may correlate with the expression of PDGFR, resulting in poor clinical outcomes. However, elevating the expression of targeted genes is also a potential therapeutic strategy for overcoming multidrug resistance. One study reported that masitinib reverses DOX resistance in canine lymphoma cells by inhibiting P-gp activity [19]. Therefore, TOC may have the potential to reverse drug resistance in refractory T-cell lymphoma in dogs.

The clinical data showed that the overall response rate (ORR) to TOC was 40% (2/5) in subjects with MDR lymphoma, and
these two subjects had PFIs of 105 and 84 days without severe adverse events or discontinuation. One study reported that the ORR to masitinib was 70% in canine epitheliotropic T-cell lymphoma. In the same study, the median PFI of the subjects that exhibited CRs was 85 days, and that of the subjects that exhibited PRs was 60.5 days [6]. In this clinical trial, it was suggested that TOC might be more effective against T-cell lymphoma than B-cell lymphoma. Moreover, expression of the TOC-targeted genes was greater in the two subjects that exhibited PRs than in the other subjects. To reinforce the suggestion that expression levels of these genes may allow estimation of therapeutic effects, further study is required to demonstrate phosphorylation levels of the receptor tyrosine kinases and related signal transduction pathways. This would include the analysis of phosphoprotein expression or phosphorylation activity by kinase inhibitors.

After failure of standard multidrug therapy, a rescue protocol based on CCNU is generally beneficial to dogs with MDR lymphoma, as CCNU is a DNA-targeted alkylating agent that does not affect the activity of P-gp [9]. One study has reported that the response of canine subjects with MDR lymphoma to CCNU lasted for a median of 86 days [9]. CCNU caused chronic neutropenia and cumulative hepatotoxicity in dogs with cancer [9], and TOC induced dose-dependent acute gastrointestinal toxicity, mild neutropenia and a rare protein-losing syndrome [13]. However, when TOC was administered (2.75 mg/kg, orally, every second day) in combination with CCNU (50 mg/m², orally, every 3 weeks) in phase I of the study, it was well tolerated, and two out of three subjects exhibited a CR or PR [13]. Hence, a combination of TOC and CCNU may improve clinical outcomes in...
MDR canine lymphoma.

The clinical data suggested that expression of PDGFRα was greater in subjects with MDR T-cell lymphoma than in those with untreated T-cell lymphoma, and that c-KIT expression was greater in subjects with T-cell lymphoma than in those with B-cell lymphoma. Our findings demonstrated that TOC monotherapy was well tolerated without severe adverse events, and two of the five subjects with MDR lymphoma exhibited PRs. The data presented here could contribute to the assessment of TOC-based therapy for canine lymphoma. To improve clinical outcomes, further research is required on enhancing the effect of TOC by combining it with conventional anticancer drugs.

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