

Article

Preliminary Metabolism of Lomustine in Dogs and Comparative Cytotoxicity of Lomustine and Its Major Metabolites in Canine Cells

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Abstract: The nitrosourea drug lomustine is used clinically for treating a wide variety of malignancies, most commonly brain tumors and lymphoma. Lomustine undergoes hydrolysis *in vivo* to form isomeric metabolites, primarily trans-4-hydroxylomustine (trans-4) and cis-4-hydroxylomustine (cis-4) in various animal species including humans. Despite its widespread usage to treat canine lymphoma, the metabolism of lomustine has not been studied in dogs. It is reported that 4'-hydroxylation products of lomustine (trans-4 and cis-4) have enhanced alkylating activity and reduced toxic effects relative to lomustine, resulting in a better therapeutic index of each of the metabolites relative to the parent compound. Our results show that the metabolic profile of lomustine in dogs is similar to that in humans with trans-4 being the major metabolite and cis-4 as the minor metabolite. Comparative cytotoxicity studies of lomustine and its trans-4 and cis-4 metabolites in canine lymphoma cell lines 17–71 and GL-1 show that there is no difference in the cytotoxicity of the three compounds. In addition, a concentration and time-dependent cell killing was seen in both of these cell lines. Also, primary canine cells like peripheral blood mononuclear cells (PBMC) from lymphoma dogs did not show any sensitivity towards lomustine and its metabolites.

Keywords: Lomustine; trans-4-hydroxylomustine; cis-4-hydroxylomustine; lymphoma; cytotoxicity

Abbreviations

PBMC	Peripheral blood mononuclear cells
RBC	Red blood cells
VC	Vehicle Control
PAC-1	Procaspase-3 activating compound

1. Introduction

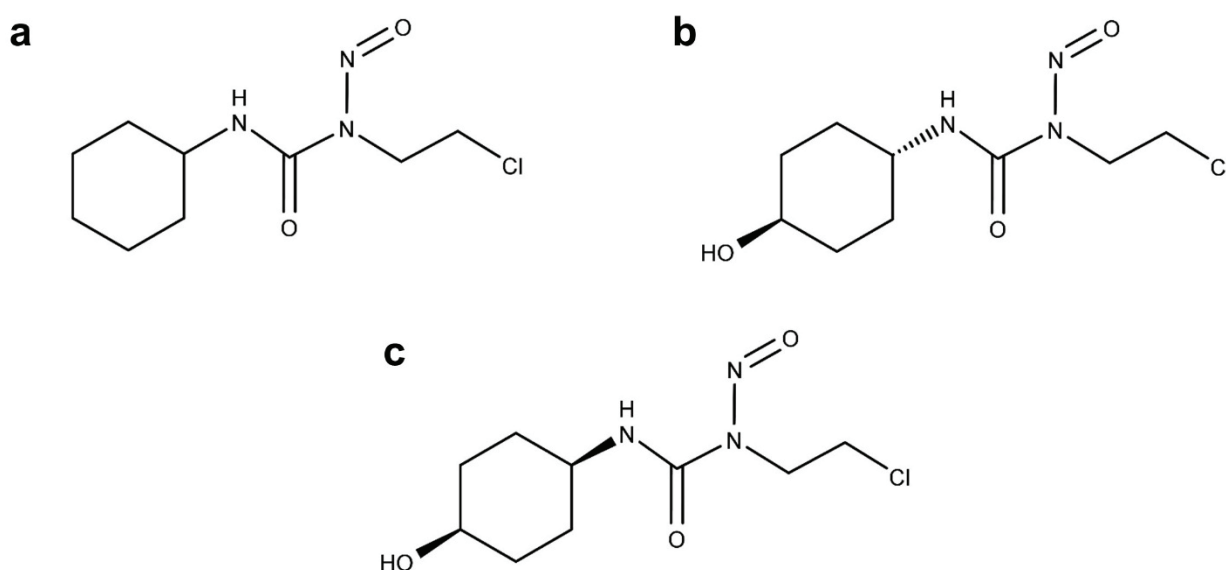
Lymphoma is one of the most common hematopoietic neoplasm seen in dogs, accounting for 7–24% of all cancers diagnosed in canines [1]. Multicentric lymphoma is the most common anatomic form accounting for over 80% of all diagnosed cases [2]. A common manifestation of multicentric lymphoma is the development of non-painful generalized lymphadenopathy with peripheral lymph nodes often bilaterally and symmetrically enlarged [3]. The treatment approach for canine lymphoma is determined by the stage of the disease. The current standard of care for dogs with lymphoma involves the use of multiagent chemotherapy, which achieves response rates of 65% to 96% and first-remission durations of 6 to 9 months [4]. There are various multiagent chemotherapy protocols such as different variations of ‘CHOP’ (cyclophosphamide, doxorubicin, vincristine, prednisolone). Despite favorable treatment outcomes initially, the majority of dogs will experience relapse, necessitating the reinstitution of additional chemotherapies for the management of refractory disease. Second remission is harder to achieve and may be of shorter duration [5]. Some relapsed dogs become refractory to the initial treatment and hence may require a different chemotherapeutic agent/rescue therapy to achieve second remission. For rescue therapy, again a multiagent protocol is followed; however, a single agent therapy may be preferred for clients with financial or logistical restrictions. Also cross-resistance between chemotherapeutic agents can decrease the efficacy of rescue protocols. Alkylating agents seldom show cross-resistance and are quite effective in achieving clinical remission [6]. Alkylating agents include chemotherapeutic drug classes such as nitrosoureas, nitrogen mustards, and alkyl sulfonates, and they function by attaching an alkyl group to the DNA.

The nitrosourea drug, lomustine (CCNU, CeeNU, $C_9H_{16}ClN_3O_3$, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea), is used clinically for treating a wide variety of human malignancies, most commonly brain tumors and lymphoma [7,8]. In veterinary medicine, lomustine has been successfully used primarily for the treatment of resistant lymphoma [5,9,10], but also for the treatment of mast cell tumors [11], intracranial meningioma [12], epitheliotropic lymphoma [13], and histiocytic sarcoma [14] in dogs either alone or in combination with other chemotherapeutic agents. There are several studies in the literature indicating good efficacy of lomustine in combination with other chemotherapeutic agents such as L-asparaginase, doxorubicin, *etc.* for the treatment of resistant canine lymphoma [5,9,10]. However, its potential as a single agent for first line therapy or to treat relapsed canine lymphoma is

less promising. Lomustine as a single agent may be preferred when treatment costs prohibit exploring other options or when a multidrug treatment is not tolerated by the patient due to extreme side effects.

In a clinical setting, lomustine is usually administered to dogs orally at 50–90 mg/m² every three weeks [5,10,15]. Lomustine is a highly lipophilic nitrosourea compound, which undergoes hydrolysis *in vivo* to form isomeric metabolites, primarily trans-4-hydroxylomustine (trans-4) and cis-4-hydroxylomustine (cis-4) (Figure 1) in various animal species including humans [16–18]. The comparative biological effects of lomustine and its metabolites were studied in mice. The single dose lethal dose (LD)₁₀ (dose causing death of 10% of the mice when administered intraperitoneally) and single dose effective dose (ED)₅₀ (dose producing 45 day survival in 50% of the mice after intraperitoneal implantation of 10⁵ L1210 cells) were compared to determine the therapeutic index for each compound [19]. The metabolites showed an approximately two-fold greater therapeutic index compared to the parent drug. It was therefore suggested that hydroxylation at the 4'-position of the cyclohexyl ring of lomustine, confers both isomers (cis-4 and trans-4) of the metabolite with enhanced alkylating activity and reduced toxic effects relative to lomustine, resulting in better therapeutic index of each of the metabolites relative to the parent compound [19].

Figure 1. Chemical structure of lomustine (a), trans-4-hydroxylomustine (b) and cis-4-hydroxylomustine (c).



Hence it is plausible that the metabolites of lomustine could be better options for chemotherapy than the parent drug, with greater efficacy and a more favorable side effect profile. In addition to the above metabolites, minor metabolites are also formed due to hydroxylation at the second and third position of the cyclohexyl ring [16,19,20]. The primary objectives of this study were: (1) to determine the preliminary metabolism characteristics of lomustine in dogs; (2) to evaluate the comparative cytotoxicity of lomustine and its trans-4 and cis-4 metabolites in canine lymphoma cell lines 17–71 and GL-1 and also in peripheral blood mononuclear cells (PBMC) from lymphoma dogs.

2. Materials and Methods

2.1. Animals and Sample Collections

The *in vivo* metabolism characteristics of lomustine were studied in two healthy research hounds. The dogs received 1.7 mg/kg (53.6 mg/m²) oral administration of commercially available lomustine capsules, and blood samples were collected at 15, 30, 45 min, 1, 2, 4, 6, 8 and 12 h after drug administration. Samples were placed in EDTA microcentrifuge tubes and kept on ice until centrifugation (10 min 5,000 g). Additionally, lomustine was also administered to a dog with lymphoma. The dog was presented at the veterinary teaching hospital and was newly diagnosed for lymphoma. No signs of any concurrent illness were noted in the dog. Lomustine and its metabolites were analyzed in the plasma samples immediately using the high performance liquid chromatography (HPLC) method described below. All study procedures were approved by the university's Institutional Animal Care and Use Committee.

2.2. Analytical Detection of Lomustine and Its Monohydroxylated Metabolites

The samples were analyzed using a peer-reviewed and published analytical method described elsewhere [21]. Briefly, a standard solution of 1 mg lomustine (Sigma-Aldrich Co., St. Louis, MO, USA), trans-4 and cis-4 was prepared in 1 mL HPLC grade ice-cold methanol. Standards were prepared by the addition of a specified amount of lomustine, trans-4, and cis-4 in ice-cold methanol to blank canine plasma samples, 0.5 mL each, over a range from 25 to 5,000 ng/mL. Phenytoin (Sigma-Aldrich Co., St. Louis, MO, USA) was used as an internal standard. Clean 16 × 150 mm screw-cap culture tubes with polytetrafluoroethylene cap liners (Fisher Scientific, Pittsburg, PA, USA) were silanized with trimethylchlorosilane (TMCS) before use by rinsing them first with methanol (MX0488-1, EMD Chemicals, Gibbstown, NJ, USA), followed by a 5% solution of TMCS in hexane (Fisher Scientific, Pittsburg, PA, USA). This step was followed by rinsing with methanol (MX0488-1, EMD Chemicals, Gibbstown, NJ, USA) twice. The silanized tubes were allowed to air-dry at room temperature. The analytical method used was as follows: Plasma samples (0.5 mL) were pipetted into 15-mL screw-cap tubes. Twenty microliters of 100 ng/μL internal standard were added to each tube, followed by 2 mL of dichloromethane. The tubes were then vortex mixed for a few seconds, then centrifuged at 2,000 rpm for 15 min. The top aqueous layer was discarded off and the organic layer was transferred into clean tubes and dried under a stream of nitrogen at 20 °C in a water bath. The residue was reconstituted with 120 μL of ice-cold methanol, vortex mixed briefly, and then transferred to an autosampler vial equipped with a 300 μL spring-loaded insert. All samples were analyzed within 24 h post-extraction.

The HPLC procedure was adapted from that described elsewhere [18]. The instrument employed was HP 1100 HPLC system with quaternary solvent delivery pumps, vacuum degasser, thermostated column compartment, 1100 series photodiode array detector, and 1100 series autosampler (Agilent Technologies, Santa Clara, CA, USA). The column was an Inertsil ODS, 5 μm particle size, 4.6 mm × 250 mm column size. The mobile phase consisted of 34% acetonitrile in water (Solvent A), 44% acetonitrile in water (Solvent B), and 64% acetonitrile in water (Solvent C) run with a flow rate of 0.8 mL/min. Samples were eluted by running two-step linear gradient commencing at the time of

injection, with an initial condition of solvent A and proceeding to solvent B within 6 min, and then to solvent C within 8 min with a total running time of 30 min. Acetonitrile (A998-4) (Fisher Scientific, Pittsburg, PA, USA) and methanol (MX0488-1, EMD Chemicals, Gibbstown, NJ, USA) were HPLC grade. The diode array detector was set up for single wavelength acquisition at 254 nm with a 12 nm span. Injections were made with a 20 µl sample loop [21].

2.3. RBC Partitioning of Lomustine and Its Metabolites

Certain drugs that have greater lipophilicity may penetrate red blood cells (RBCs) where it can be stored temporarily and this may bring down the plasma concentration of the drugs. Hence studying the RBC partitioning of drugs can help to select the appropriate matrix among whole blood, plasma or serum for assaying pharmacokinetic behavior of the drug [22]. For this study, commercially purchased canine whole blood in EDTA (The Veterinarians' Blood Bank) was used. Two sets of blood samples (four samples in each set, 1 mL/sample) were spiked with lomustine, trans-4 and cis-4 at the concentration of 500 ng/mL. After incubating the samples at 37 °C for 1 h, one set of blood samples was directly analyzed for drug concentration and in the other set, the plasma was separated and the drug concentration was measured in plasma.

The concentration in RBCs was calculated as:

$$C_{\text{RBC}} = \frac{[(C_{\text{blood}} - C_{\text{plasma}}) \times (1 - \text{Hematocrit})]}{\text{Hematocrit}}$$

where C_{blood} and C_{plasma} are the concentrations of the drug in blood and plasma, respectively.

Further, the extent of partitioning of the drugs was measured by the following formula:

$$K_{e/p} = \frac{\text{Concentration of drug in RBC}}{\text{Concentration of drug in plasma}}$$

2.4. Cytotoxicity of Lomustine and Its Metabolites in Canine Lymphoma Cell Lines and PBMC

The canine lymphoma cell lines 17-71 [23,24] and GL-1 [25] and PBMC obtained from lymphoma dogs were used for this assay.

2.4.1. PBMC Isolation

Blood samples were collected from three lymphoma dogs that were brought to the veterinary teaching hospital. The dogs were newly diagnosed cases of lymphoma and did not have any concurrent illnesses. Four milliliters of whole blood were collected into one 5 mL heparinized tubes. The procedure for isolation of PBMC from lymphoma dogs was as described by Lavergne *et al.* [26]. Tubes were placed on ice immediately, and PBMC were isolated using density centrifugation. Blood was kept on ice, and cell isolation was begun within 30 min of venipuncture. Whole blood was diluted with two volumes of prewarmed (37 °C) HBSS with 5 mM EDTA. This was carefully overlaid on 5 mL of prewarmed (37 °C) Lymphocyte Separation Media (Cellgro). After centrifugation, the buffy coat was harvested and washed with three to four volumes of Hank's balanced salt solution (HBSS) with 5 mM EDTA. Lysing buffer (2 mL; 15.5 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA, pH 7.4) was added to

pelleted cells to lyse the residual erythrocytes. After washing with HBSS with 5 mM EDTA, cells were re-suspended in PBS and an aliquot was used to quantitate viable cells using Trypan blue dye exclusion.

2.4.2. Cytotoxicity Assays

Cytotoxicity was evaluated after 48 h by XTT cell proliferation assay and Annexin-PI assay (flow cytometry). The cell lines 17–71 and GL-1 were grown and maintained at 37 °C in 5% CO₂, and cultures were passaged as necessary to maintain high cell viability (above 90%) in culture media Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. The PBMC were maintained in culture media RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin. The cell viability was assessed by Trypan blue dye exclusion test. For the XTT assay, the cells were plated in 96 well plates at a density of 20,000 cells per well. The cells were exposed to fresh media (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin for the cell lines and RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin for PBMC) containing varying concentrations of lomustine, trans-4, or cis-4 metabolites (300, 1,000, 3,000, and 10,000 ng/mL). Staurosporine (1 µM for 24 h) was used as positive control for apoptosis in GL-1 cells and PBMC while PAC-1 (procaspase-3 activating compound, 10 µM for 48 h) was used as positive controls for apoptosis in 17–71 cells. The positive controls were chosen based on initial experimentation. Staurosporine produced more than 60% apoptosis in GL-1 cells, however it was not as effective (<30% apoptosis) in 17–71 cells. Additionally, a previously published study by Lucas *et al.* [27] had shown that 17–71 cells were sensitive to PAC-1 (>70% apoptosis) but the GL-1 cells were not as susceptible (<40% apoptosis). The vehicle control (VC) was 0.1% methanol corresponding to the concentration of methanol present in each of the drug treated samples. Forty-eight hours after drug exposure, the cytotoxicity was measured using XTT Cell Proliferation Assay Kit (Promega, Madison, WI, USA). For the Annexin/PI assay, 100,000 cells were plated per well in a 12-well plate. The drug treatment protocol was similar to that in the XTT assay. In addition to the apoptosis controls, a necrosis control was also used which was Triton-X (0.03% for 10 min). At the end of 48 h of incubation, the cells were stained using the Annexin V-FITC Apoptosis detection Kit (BD Biosciences, San Jose, CA, USA) and analyzed by flow cytometry. The experiments were done three separate times in the lymphoma cell lines with two technical replicates used for each assay. The experiment was done on PBMC obtained from three lymphoma dogs ($n = 3$) with two technical replicates for each assay.

2.5. Time Dependent Cytotoxicity of Lomustine and Its Metabolites

In this experiment, the cytotoxicity of lomustine, trans-4 and cis-4 was measured in canine cell lines 17–71 and GL-1 at 48, 96, and 144 h after drug exposure in order to see if there was a time dependent cytotoxic effect of these drugs. The cells were treated with the drugs similarly as mentioned above, and apoptosis was measured by XTT and Annexin/PI assay at the end of 48, 96, and 144 h of incubation. During incubation, half the media in each well was aspirated every 48 h and replaced with an equal volume of fresh media (without drug) very carefully in order to not disturb the cells at the bottom. This was done in order to avoid acid build up in wells during the long incubation time especially in the vehicle control and at lower concentrations of the drugs where most of the cells would be viable.

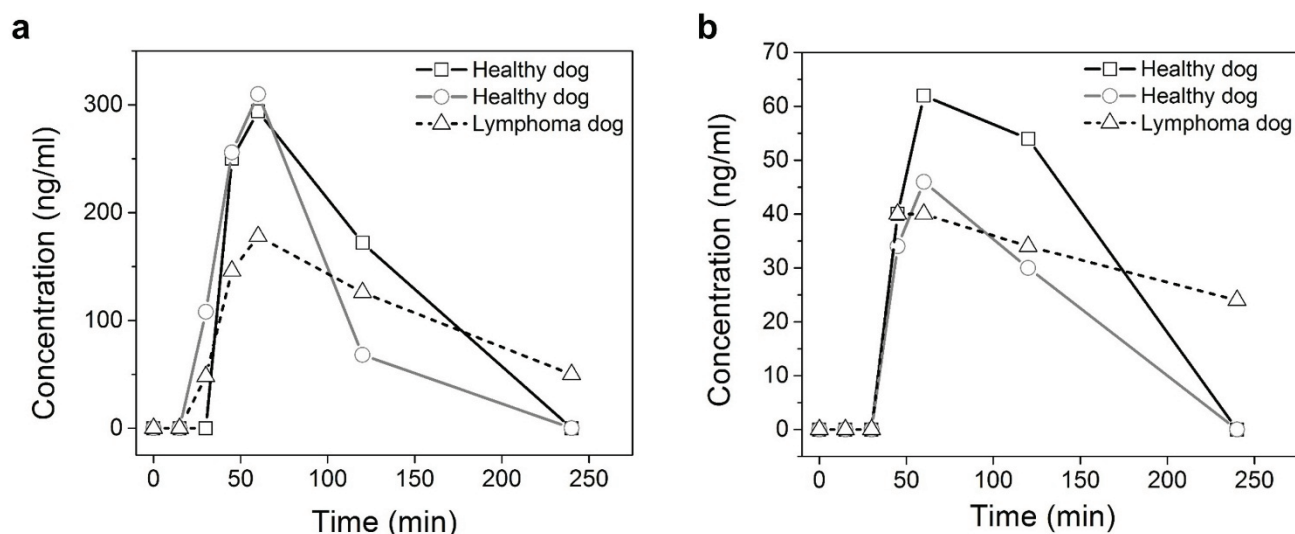
2.6. Statistical Analysis

Statistical analysis was done using SPSS software. Descriptive statistics were produced for all continuous variables. Mean and standard deviation were calculated. Normality of data was assessed using the Shapiro-Wilks test. Paired t-tests were performed to evaluate differences between technical repeats of each drug and dose combination. Multiple one way ANOVA and Tukey's *post hoc* test were performed to evaluate the following within and between group differences: Between drugs within each dose in each cell type at each time point, between doses (concentrations) within each drug in each cell type at each time, between time points within dose in cell types for the each drug. The threshold for statistical significance was set at a *p* value of 0.05. For the canine lymphoma cell lines, two out of seventy two groups did not show normal distribution using Shapiro-Wilks test. However when these groups were further evaluated for normality using kurtosis, skewness, and Q-Q plots, the data showed normal distribution. Hence a parametric test was used to reduce the probability of type II error. *Power analysis*: Cytotoxicity data from two canine lymphoma cell lines were used to estimate sample size requirements for PBMC. Using the data from the 17-71 and GL-1 cell lines, based on *a priori* information of alpha = 0.05, power of 0.8 and an expected difference in mean of 3.77 (corresponding to the maximum standard deviation seen at any concentration), we would require 3 three samples. The lowest concentration of 300 ng/mL was excluded from the power analysis as no statistically significant cell killing was seen in either of the cell lines with any of the drugs at that concentration.

3. Results

Lomustine was not detected in plasma samples of the three dogs following oral administration of the drug. Figure 2a shows that trans-4 is the major metabolite of lomustine and the maximum concentration was about 325 ng/mL detected 1 h after drug administration for both the research hounds. The maximum concentration of cis-4 in the three dogs was around 40–65 ng/mL (Figure 2b).

Figure 2. Plasma concentration of trans-4-hydroxylomustine (a) and cis-4-hydroxylomustine (b) following single 1.7 mg/kg oral administration of lomustine in three dogs.



The results show that there is a minor partitioning of these drugs into/onto RBC (Table 1). About 9–15% of the drug was found to be in the RBC compartment. For our metabolism studies, drug concentrations were measured in plasma and not whole blood. This small difference in the drug levels in plasma and whole blood could have led to a slight underestimation of the actual drug concentrations in blood.

Table 1. *In vitro* concentrations of lomustine, trans-4-hydroxylomustine and cis-4-hydroxylomustine (ng/mL) in whole blood, plasma and red blood cells.

Drug	Plasma mean \pm SD	Whole blood mean \pm SD	RBC \pm SD	$K_{e/p}$
Lomustine	287.18 \pm 27.42	309.87 \pm 14.03	37.49 \pm 4.16	0.12
Trans-4-hydroxylomustine	256.42 \pm 41.13	273.38 \pm 16.11	27.71 \pm 5.01	0.10
Cis-4-hydroxylomustine	228.98 \pm 12.40	253.81 \pm 15.90	40.75 \pm 5.66	0.17

* All samples spiked at 500 ng/mL. Hematocrit for the blood sample was 0.38 (38%). $K_{e/p}$ is the red blood cell (RBC) partitioning coefficient (Ratio of concentration of drug in RBC to plasma).

The results obtained from the XTT assay (Figure 3) and annexin/PI assay (Figure 4) in the 48 h cytotoxicity study show that there is a statistically significant cell killing at 3,000 and 10,000 ng/mL concentrations, when comparing to control for lomustine, trans-4 and cis-4 metabolites in canine lymphoma cell lines 17–71. In the canine lymphoma cell line GL-1, in addition to the above concentrations, 1,000 ng/mL also showed significant cytotoxicity compared to the VC. After treating the cells with the highest drug concentration, 10,000 ng/mL, about 50% and 40% of the cells were still viable at 48 h in 17–71 and GL-1 cells, respectively (Figure 4). The results obtained from both XTT and Annexin/PI assay do not show any statistically significant difference in the cytotoxicity caused by lomustine, trans-4, and cis-4 in 17–71 and GL-1 cells at 48 h. Lomustine and its metabolites did not show any cell killing in the PBMC obtained from three lymphoma dogs at 48 h after exposure to lomustine, trans-4, and cis-4 (Figure 5). Our results show that terminally differentiated lymphocytes in circulation may not be as sensitive to alkylating agents as lomustine at 48 h. It is possible that PBMC may require a longer incubation time for the cytotoxicity of lomustine to become apparent.

In the time-dependent cytotoxicity study, all three compounds showed a greater degree of cell killing at 96 and 144 h as compared to 48 h, thus confirming the premise that cytotoxicity with these drugs is delayed in nature (Figure 6). However, no statistically significant difference was seen in the cytotoxicity among the three compounds. In the 17–71 cell, the 300 ng/mL samples did not show any cell killing or reduction in viability even at 144 h after drug treatment. The 3,000 and 10,000 ng/mL concentrations resulted in a comparable level of cell killing at 144 h. Also, using 10,000 ng/mL at 96 h data, more than 60% of the cells were non-viable. Due to this ‘saturation’ in cell killing, very little difference was seen in cytotoxicity at 144 h as compared to 96 h at 10,000 ng/mL. These results show that there is a delay in cell killing by lomustine and its metabolites, and hence measuring cell viability/apoptosis after 48 h of incubation may not be the best indicator of the cytotoxic potential of the compounds.

Figure 3. Graphs showing the results of XTT assay in 17-71 (a) and GL-1 (b) cells at 48 h. Vehicle control (VC) was 0.1% methanol. The results show the mean and standard deviation of three independent experiments ($n = 3$).

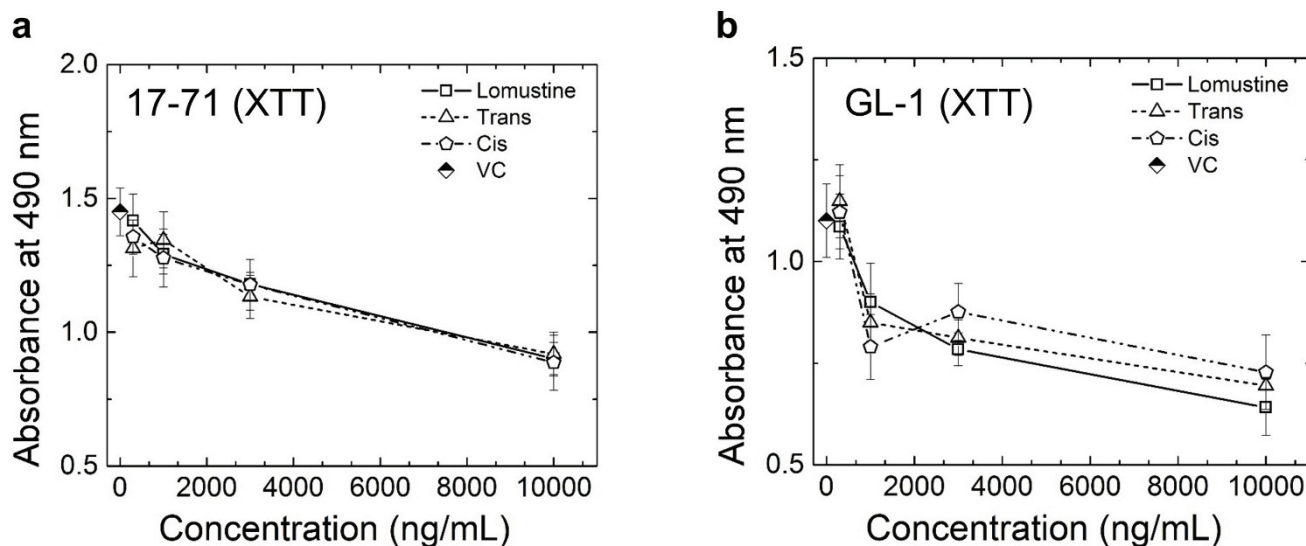


Figure 4. Graphs showing the percent viable cells obtained from Annexin/PI assay in 17-71 (a) and GL-1 (b) cells at 48 h. Vehicle control (VC) was 0.1% methanol. The results show the mean and standard deviation of three independent experiments ($n = 3$).

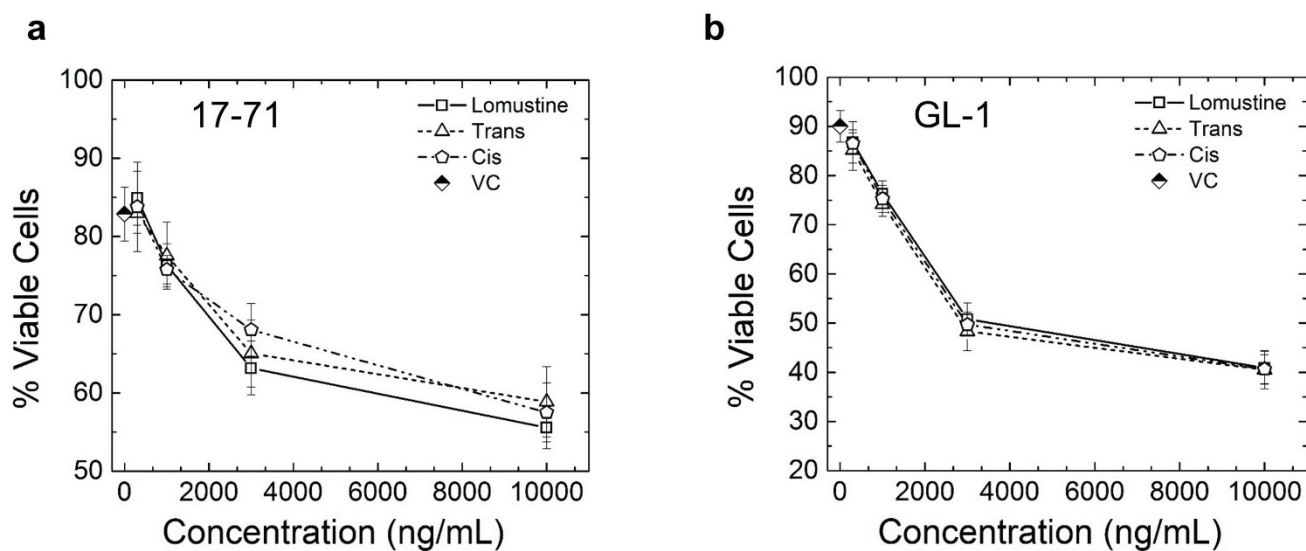


Figure 5. Graphs showing the results of XTT assay (a) and Annexin/PI assay (b) in peripheral blood mononuclear cells (PBMC) isolated from three lymphoma dogs. Vehicle control (VC) was 0.1% methanol. The graph shows the mean and standard deviation of results obtained from three dogs. No statistically significant difference was seen in any of the drug treated samples compared to VC.

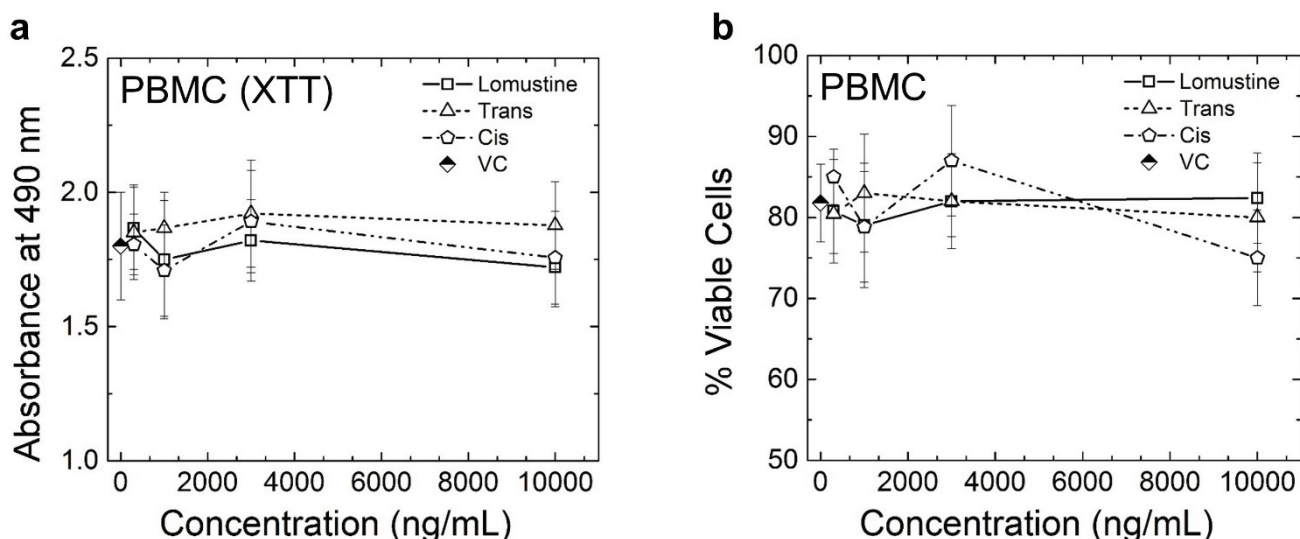
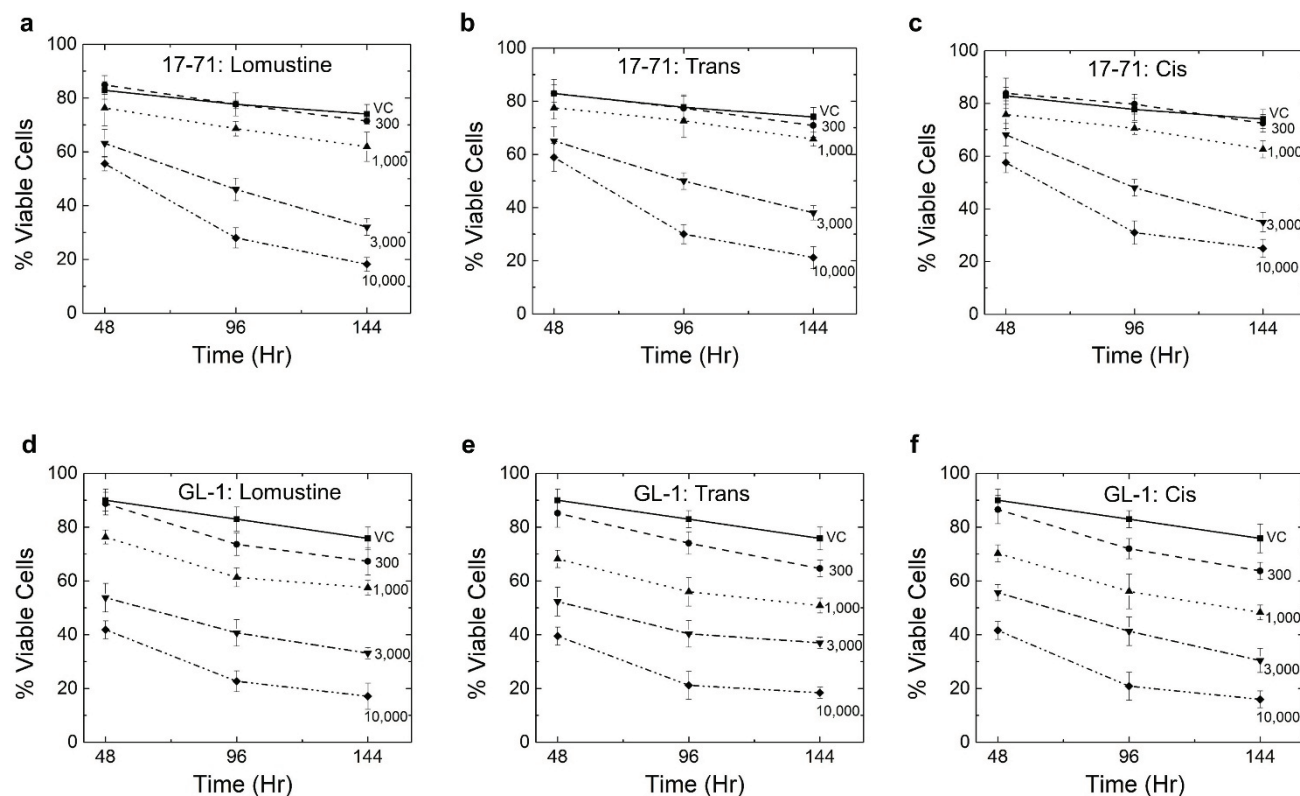


Figure 6. Graphs showing the results of Annexin/PI assay in 17-71 cells (a, b and c) and GL-1 cells (d, e, and f) at 48, 96 and 144 h after treatment with lomustine, trans- and cis-4-hydroxylomustine. Vehicle control (VC) was 0.1% methanol. The results show the mean and standard deviation of three independent experiments (n = 3).



4. Discussion

Lomustine has been identified as a potentially important therapeutic agent for use in the treatment of various cancers in dogs; as such, we needed a sensitive and reliable analytical method to perform pharmacokinetic experiments with this agent in dogs. In particular, the method selected needed to be sufficiently sensitive to detect low concentrations of lomustine and its possible monohydroxylated metabolites in canine plasma from test animals.

The resultant HPLC method is a sensitive analytical method with the LOD of lomustine, cis-4 and trans-4 in plasma of about 10 ng/120 μ L, 5 ng/120 μ L, and 5 ng/120 μ L, respectively, based on signal to noise ratio. Additionally, satisfactory recovery was obtained for lomustine and its monohydroxylated metabolites extracted from plasma samples. The developed extraction procedure is very simple (one step liquid/liquid extraction procedure) and suitable to analyze multiple samples in a short-time period. The CV for within-run and between-run samples were less than 15% for lomustine, trans-4, and cis-4, indicating good method precision [21].

The metabolic profile of oral lomustine in dogs established in our study is similar to that reported in humans and rats with trans-4 being the major metabolite followed by cis-4 and the complete absence of parent lomustine. However, since the study was only done in three dogs, these results have to be considered as preliminary. In our study, the dogs received lomustine at the dose of 1.7 mg/kg, and the two research hounds showed similar peak levels of trans-4 (about 325 ng/mL), but the level for the same in the lymphoma dog was lower. The peak cis-4 lomustine level was between 40–65 ng/mL. In a published study of four human patients that received lomustine at the dose of 4.1 mg/kg, parent lomustine was not detected in the blood. The average peak concentration of trans-4 was 530 ng/mL and that for cis-4 was 320 ng/mL [18]. In another study in human patients that used high dose lomustine (15 mg/kg), the peak plasma concentration detected ranged between 410–2,310 ng/mL for trans-4 and 180–1,820 ng/mL for cis-4 [17], a five- and 10-fold difference between the maximum concentrations observed among patients, respectively. This supports the premise that there is a large inter-individual difference in the metabolic profile of lomustine.

As parent lomustine was not detected in the blood at any time after administration of the drug, the RBC partitioning behavior of lomustine and its metabolites was investigated. The results showed that there is a minor partitioning of these drugs into RBC. However, RBC partitioning values were not high enough to explain why lomustine was not detected in our canine plasma samples. It is likely that, like in other animal species, the absence of lomustine in dogs' plasma is due to rapid "first pass" metabolism and not due to significant RBC partitioning.

As mentioned previously it has been reported that the major hydroxylated metabolites of lomustine may have enhanced alkylating activity and reduced toxic effects relative to parent lomustine [19]. In this study, we wanted to investigate whether the major hydroxylated metabolites showed potential as a more effective chemotherapeutic option than lomustine using *in vitro* studies. The metabolism studies discussed show that trans-4 and cis-4 are the major metabolites in dogs after oral treatment with lomustine, with trans-4 being in greater proportion.

The drug concentrations for the cytotoxicity study were derived from the metabolism studies in dogs where we found that the maximum plasma concentration of trans-4 was about 300 ng/mL and that for cis-4 was 50 ng/mL. Hence we used concentrations starting at 300 ng/mL up till 10,000 ng/mL

as we did not know at what concentration the *in vitro* biological effects of the drugs would be seen. We found no significant difference in the cytotoxicity caused by lomustine and its trans-4 and cis-4 metabolites in both the canine lymphoma cell lines 17–71 and GL-1. At 48 h after treatment with 10,000 ng/mL lomustine or its metabolites, 40–50% of the cells were still viable. However, after 96 h of incubation this number had dropped to about 20% indicating a time-dependent effect of lomustine cytotoxicity. In the PBMC from three lymphoma dogs, none of the drugs showed any significant cell killing compared to the control at 48 h after lomustine treatment. In two of the dogs, there were no circulating lymphoma cells detected. Hence the cytotoxicity results in PBMC of those two patients should represent the sensitivity of “normal” lymphocytes to the three agents. However, the third lymphoma patient had a lot of circulating lymphoma cells (37% of all of the nucleated cells). Hence, for this patient, it is highly probable that when we screened the sensitivity of “normal lymphocytes”, we were indeed also screening a good proportion of malignant lymphoma cells too. This would make interpretation of results from this patient more difficult and problematic. Interestingly, the *in vitro* inhibitory concentration (IC)₅₀ for lomustine mentioned in literature varies from 50–250 μ M (11,650–58,400 ng/mL) [28–30]. These studies have utilized different cell lines and also used different techniques of detecting the cell killing. However, a time-dependent study of lomustine cytotoxicity has not been done before. Our findings suggest that apoptosis of cells after lomustine administration is not an immediate event but rather a delayed one. Also, the therapeutic concentration of lomustine measured in the three dogs (about 300 ng/mL) did not show any cytotoxic effect in the *in vitro* study with the canine cell lines even at 144 h (6 days) of incubation. One reason for this disparity could be that since lomustine is metabolized rapidly to form pharmacologically active metabolites and also spontaneously breaks down into reactive intermediates which ultimately cause the alkylation of DNA, measuring the parent compound alone may not indicate the extent of the cytotoxic effect of the drug. The anticancer effect of lomustine may be brought about by the combined action of lomustine and its active metabolites whose absolute quantification in blood is precluded by their rapid breakdown in physiological body conditions.

5. Conclusion

In conclusion, our previously published analytical method have shown that lomustine and its metabolites are highly unstable and that careful consideration must be given to the stability of these compounds while storing and using them for experimental purposes [21]. In addition, we have shown that the metabolism profile of lomustine in dogs is similar to that in humans. We also conclude that there is no difference between the cytotoxicity of lomustine, trans-4, and cis-4 in the two canine lymphoma cell lines 17–71 and GL-1 and that cell killing by all three compounds appears to be time-dependent. Finally, lomustine and its metabolites did not significantly reduced the viability of canine primary cells like PBMCs suggesting that a much longer exposure time or delay after exposure is required to significantly kill primary leukocytes in dogs.

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Authors Contributions

All the authors included in this study contributed to study design, data analysis, sample collection, and manuscript preparation.

Conflict of Interest

The authors declare no conflict of interest.

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