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Pulse-Administered Toceranib Phosphate Plus Lomustine for Treatment of Unresectable Mast Cell Tumors in Dogs

J.H. Burton, R.O. Venable, D.M. Vail, L.E. Williams, C.A. Clifford, S.M. Axiak-Bechtel, A.C. Avery, and D.H. Thamm

Background: Nonresectable mast cell tumors (MCT) in dogs remain a therapeutic challenge, and investigation of novel combination therapies is warranted. Intermittent administration of tyrosine kinase inhibitors (TKI) combined with cytotoxic chemotherapy may effectively chemosensitize canine MCT while decreasing cost and adverse effects associated with either agent administered as monotherapy.

Hypothesis/Objectives: The primary study objectives were to (1) identify the maximally tolerated dose (MTD), (2) determine the objective response rate (ORR) and (3) describe the adverse event profile of pulse-administered toceranib phosphate (TOC) combined with lomustine.

Animals: Forty-seven client-owned dogs with measurable MCT.

Methods: Toceranib phosphate was given PO on days 1, 3 and 5 of a 21-day cycle at a target dosage of 2.75 mg/kg. Lomustine was given PO on day 3 of each cycle at a starting dosage of 50 mg/m^2. All dogs were concurrently treated with diphenhydramine, omeprazole, and prednisone.

Results: The MTD of lomustine was established at 50 mg/m^2 when combined with pulse-administered TOC; the dose-limiting toxicity was neutropenia. Forty-one dogs treated at the MTD were evaluable for outcome assessment. The ORR was 46% (4 complete response, 15 partial response) and the overall median progression-free survival (PFS) was 53 days (1 to >752 days). On multivariate analysis, variables significantly associated with improved PFS included response to treatment, absence of metastasis, and no previous chemotherapy.

Conclusions and clinical importance: Combined treatment with pulse-administered TOC and lomustine generally is well tolerated and may be a reasonable treatment option for dogs with unresectable or metastatic MCT.

Key words: Cancer; Chemotherapy; Dog; Tyrosine kinase inhibitor.

From the Flint Animal Cancer Center, Department of Clinical Sciences, Colorado State University, Fort Collins, CO (Burton, Venable and Thamm); the School of Veterinary Medicine and the Carbone Cancer Center, University of Wisconsin-Madison, Madison, WI (Vail); the Department of Clinical Sciences, North Carolina State University, Raleigh, NC (Williams); the Red Bank Veterinary Hospital, Tinton Falls, NJ (Clifford); the Department of Veterinary Medicine and Surgery, University of Missouri, Columbia, MO (Axiak-Bechtel); and the Flint Animal Cancer Center, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO (Avery); Dr. Burton's present address is Department of Surgical and Radiological Sciences, University of California, Davis, Davis, CA; Dr. Venable's present address is Arizona Veterinary Oncology, Gilbert, AZ; Dr. William's present address is Veterinary Specialty Hospital of the Carolinas, Cary, NC; Dr. Clifford's present address is Hope Veterinary Specialists, Malvern, PA.

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Patients were enrolled at the Flint Animal Cancer Center, Colorado State University Veterinary Medical Center (CSU-VMC), School of Veterinary Medicine, University of Wisconsin-Madison (UW-SVM), Veterinary Medical Teaching Hospital, University of Missouri (MU-VMTH), Red Bank Veterinary Hospital and the Veterinary Health Complex, North Carolina State University (NCSU-VHC).

Corresponding author: Dr D.H. Thamm, Flint Animal Cancer Center, Department of Clinical Sciences, Colorado State University, 300 W. Drake Road, Fort Collins, CO 80523-1620; e-mail: doug.thamm@colostate.edu.

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Abbreviations:

ALT	alanine transaminase
ALP	alkaline phosphatase
CR	complete response
DLT	dose-limiting toxicity
ITD	internal tandem duplication
MCT	mast cell tumor
MTD	maximally tolerated dose
ORR	objective response rate
OS	overall survival
PR	partial response
PFS	progression-free survival
PD	progressive disease
RECIST	response evaluation criteria in solid tumors
SD	stable disease
TOC	toceranib phosphate
TTMR	time to maximal response
TKI	tyrosine kinase inhibitor
VCOG-CTCAE	Veterinary Co-operative Oncology Group – Common Terminology Criteria for Adverse Events

Mast cell tumors (MCT) are the most common cutaneous tumors in the dog and are generally successfully treated with wide surgical excision alone.[1–3] Therapeutic challenges, however, arise with MCT that are large or infiltrative, high grade, have metastasized beyond the regional lymph node, or are located where wide surgical excision is not possible. Several medical treatments have been studied for MCT in dogs, including corticosteroids alone, lomustine, chlorambucil, hydroxyurea, and vinblastine as well as various combinations of these agents.[4–11] Although generally well tolerated, response rates frequently

remain at or below 50% and usually are brief and incomplete.[4–12]

The tyrosine kinase inhibitor (TKI), toceranib phosphate (TOC), has demonstrated single-agent antitumor activity against MCT in dogs, but fewer than half of the dogs with MCT experience objective tumor regression with only 14% experiencing complete responses (CR).[13] The TKI sunitinib and radiation therapy are synergistic in preclinical models of pancreatic adenocarcinoma, soft tissue sarcoma, and breast cancer in humans[14–16] as well as between TOC and radiation therapy in dogs with MCT.[17] In addition, several studies have identified potentiation of the efficacy of paclitaxel, doxorubicin, and vincristine by the TKI imatinib in human preclinical models of KIT-positive melanoma and Ewing's sarcoma,[18–20] presumably owing to downregulation of important antiapoptotic pathways. A recent phase I study evaluating the safety of the combination vinblastine and TOC in dogs reported the necessity for substantial dose reductions of vinblastine in this combination therapy to prevent frequent and severe neutropenia.[21] Together, these data suggest that combination therapies with TKIs may improve efficacy over single-agent treatments alone.

Clinically relevant adverse effects can be observed with continuous long-term TKI administration, including diarrhea, inappetence, neutropenia, proteinuria, fatigue, and musculoskeletal pain, resulting in the need for drug holidays and dose reductions.[13,22,23] Pulse administration of TKIs with chemotherapy potentially may chemosensitize tumor cells while decreasing cost and toxicity associated with chronic TKI administration.

This phase I/II multicenter clinical trial sought to determine the maximally tolerated dose (MTD), tolerability and adverse event profile of combined treatment with pulse-administered TOC, and lomustine in dogs with measurable MCT. The second objective of this study was to determine the objective response rate (ORR), progression-free survival (PFS) and overall survival (OS) in dogs with measurable MCT treated with combined pulse-administered TOC and lomustine, and to identify potential prognostic factors in dogs treated with this combination. We hypothesized that the combination of pulse-administered TOC and lomustine would be well tolerated and efficacious when administered to dogs with cutaneous MCT.

Materials and Methods

Patient Selection

Client-owned dogs with histologically or cytologically confirmed MCT ≥10 mm in longest diameter, where surgical excision was not feasible or was declined by the owner, were considered for enrolment. Eligibility criteria included Veterinary Co-operative Oncology Group – Common Terminology Criteria for Adverse Events (VCOG-CTCAE) constitutional health score of 0 (normal activity) or 1 (mildly decreased from baseline)[24], and life expectancy of >6 weeks. Dogs were required to have adequate hematologic, renal, and hepatic function to safely undergo treatment; defined as ≥2,500 neutrophils/μL, ≥75,000 platelets/μL, PCV ≥28%, serum creatinine concentration ≤2× the upper limit of normal (ULN), total serum bilirubin concentration <1.5× ULN, alanine transaminase (ALT) activity ≤2× ULN, aspartate aminotransferase activity ≤2× ULN, and gamma-glutamyl transferase activity ≤2× ULN. Dogs were excluded if they had other serious comorbid diseases or had previously been treated with lomustine or any TKI. Prior surgery was allowable as was radiation therapy or chemotherapy (excluding previous treatment with lomustine or any TKI) with 6- and 2-weeks washout periods after radiation therapy and chemotherapy, respectively.

Baseline evaluations included medical history, physical examination, abdominal ultrasound examination, cytologic evaluation of regional lymph nodes, CBC, serum biochemistry, and urinalysis. Thoracic radiographs were not required as part of the study but were performed by some supervising clinicians as routine staging for MCT. Tumor aspirates also were obtained at the time of enrollment to assess for *c-kit* gene mutation. The participating institutions' Animal Care and Use Committees or Clinical Review Boards approved the clinical protocol, and written informed consent was obtained from the owners before patient enrollment.

Phase I Study Design and Treatment Protocol

Dogs were given diphenhydramine 2–4 mg/kg PO q12h, omeprazole 0.7 mg/kg PO q24h, and prednisone 1 mg/kg PO q48h for a minimum of 72 hours before the initiation of the treatment with TOC; these medications were continued throughout the study period. An open-label, phase I, 3 + 3 dose-cohort escalation design was employed to assess the safety of combination pulse-dosed TOC and lomustine.[25] All dogs were scheduled to receive TOC 2.75 mg/kg PO once on days 1, 3, and 5 of a 21-day cycle; the TOC dose remained the same throughout the study and was given on alternating days from the prednisone. Lomustine was administered PO once on day 3 of the 21-day cycle; a starting dosage of 50 mg/m^2 was selected for the initial cohort. The lomustine dose was approximated to the nearest 10 mg using combinations of commercially available 40 and 10 mg capsules.

Three dogs were enrolled in the first dose cohort and observed for dose-limiting toxicities (DLT). A DLT was defined as any grade 3 nonhematologic or grade 4 hematologic toxicity according to the VCOG-CTCAE v1.0.[26] If no DLTs were observed in the first cohort of 3 dogs within 3 weeks of lomustine administration, a second cohort was treated with the same dosage of TOC and an increased dosage of lomustine. If a DLT was observed in 1 dog, the cohort was expanded up to a total of 6 dogs. If no additional DLTs were noted in the expanded cohort of 6 dogs, dose escalation was continued with a higher dosage of lomustine. If ≥2 DLTs were observed in the initial or expanded cohort, case accrual was stopped and the MTD was determined to be the dosage used in previous cohort where <2 DLTs were noted. Escalation of the lomustine dosage was planned in 10 mg/m^2 increments until the MTD of the combination therapy was established.

Safety Evaluation

Dogs were evaluated 1 week after the first dose of lomustine and a physical examination and CBC were performed. Reevaluation occurred again 3 weeks after the lomustine dose; physical examination, tumor measurements, CBC, and assessment of ALT activity with or without other liver enzymes was performed at that time. Owners completed a quality of life assessment form at each study visit that has been previously described.[27] Adverse events noted on laboratory evaluation, physical examination, or noted by owners were prospectively graded using VCOG-CTCAE v1.0.[26] For dogs experiencing grade 3 or 4 neutropenia, a 20% dose reduction of lomustine was performed for subsequent dosing cycles. Increases in hepatic transaminase activity were managed at

the discretion of the attending clinician with lomustine dose reductions, delays in treatment, or both. Either prophylactic or therapeutic treatment with hepatoprotectants was also allowable.

Antitumor Response Assessment

Once a MTD was identified, cohort expansion at the MTD was performed according to a Simon's Minimax design to evaluate the efficacy of pulse-administered TOC and lomustine.[28] Twenty-eight dogs were to be enrolled in the first stage. If ≥11 of the 28 dogs initially enrolled at the MTD experienced a tumor response, the cohort was to be further expanded to include an additional 13 dogs to better define the response to treatment. If <11 of the 28 dogs experienced a response, enrollment would be discontinued.

Tumor response was assessed every 3 weeks in all dogs enrolled in this study using modified Response Evaluation Criteria in Solid Tumors (RECIST) criteria. The longest diameters of the target lesions were documented before treatment initiation with TOC. A CR was defined as the disappearance of all lesions, a partial response (PR) was defined as at least a 30% decrease in the sum of the target lesion diameters, and progressive disease (PD) was defined as a ≥20% increase in the sum of the target lesion diameters using baseline measurement as a reference. Stable disease (SD) was defined as neither CR, PR, nor PD and must have persisted for a minimum of 6 weeks. Dogs experiencing SD or PR were given pulse-dosed TOC and lomustine once every 3 weeks until disease progression. Dogs experiencing CR were treated with 5 cycles of the TOC/lomustine combination or 2 cycles beyond documentation of a clinical CR, whichever was longer, and were evaluated monthly after the last cycle of TOC/lomustine for tumor response assessment. Dogs experiencing PD were removed from the study.

c-Kit Mutation Status

Nucleic Acid Extraction. Genomic DNA samples were prepared from Wright-Giemsa-stained fine needle aspirates. Slides were confirmed to have ≥10% of the cell population as mast cells. Qiagen AL buffer was applied to the slides, which were then scraped with a straight-edged razor into microcentrifuge tubes. DNA extraction was then performed with a commercial kit according to the manufacturer's instructions.[a]

PCR Amplification of c-kit Exons 8 and 11. Regions of *c-kit* exons 8 and 11 were amplified with primers directed against sequences flanking the characterized internal tandem duplications (ITDs). The primer pair for exon 8 consisted of (TGACCTA TGGCCATTTCTCT) coupled with (56FAM-AATCCTGCAA CCACACACTG), resulting in a product of 92 bases. The pair for exon 11 consisted of (CAGTGGAAGGTTGTTGAGGAG) coupled with (VIC-CATGGAAAGCCCCTATTTCA), resulting in a product of 132 bases. Amplifications were performed with a commercially available PCR kit.[b] Primer concentrations used were 400 nM each.

Gene scanning analysis was performed using a capillary electrophoresis machine.[c] Amplified fragments were run with Gene Scan™-600 LIZ size standards. Raw data were analyzed with a commercially available genotype analysis software.[d]

Statistics

Continuous data were expressed as median and range, and categorical data as frequencies and percentages. Time to maximal response (TTMR) was calculated from the date of treatment initiation to the date the best overall response was first documented. Progression-free survival and OS were calculated from the date of administration of the first dose of TOC to the date of PD or death, respectively. Dogs that were still alive at the time of data analysis were censored at the last date reported to be alive. Dogs that had died were considered to be dead either secondary to their treatment or their disease. Kaplan–Meier estimation was used to estimate and display the distribution of the PFS and OS. Logrank and Cox proportional hazards regression was used to evaluate associations between patient and treatment factors and PFS and OS. Simple regression was first used to determine which covariates to include in the multiple regression model based on an alpha level of 10%. Covariates with P-values < 0.10 in simple regression models were then included in multiple regression models. Multiple regression models were estimated and covariates were removed in a forward and reverse stepwise fashion because of insignificance at the 5% alpha level. Variables with values of $P \leq 0.05$ were considered significant. All statistical analyses were performed using commercial software packages.[e,f]

Results

Patient Population

A total of 47 dogs were enrolled in the clinical trial from March, 2011 to March, 2013; 13 were enrolled into the dose escalation phase and the remainder into the dose expansion phase. Dogs were enrolled at the Flint Animal Cancer Center at Colorado State University (n = 28), North Carolina State University (n = 8), University of Missouri (n = 5), University of Wisconsin-Madison (n = 4) and Red Bank Veterinary Hospital (n = 2). Patient demographics and tumor characteristics for all dogs enrolled in phase 2 of the clinical trial are described in Table 1.

Dose Escalation

The dose escalation cohorts are summarized in Table 2. The MTD of lomustine when combined with pulse-dosed TOC was determined to be 50 mg/m^2. Eight dogs were enrolled in the first cohort and given lomustine at 50 mg/m^2. One of the first 3 dogs enrolled experienced a grade 4 neutropenia and 2 dogs subsequently enrolled were unable to be evaluated for safety assessment, because they were withdrawn from the study before day 7 because of progression of disease. The lomustine dosage was increased to 60 mg/m^2 in 2 dogs and both dogs in this cohort experienced grade 4 neutropenia. The dosage then was de-escalated to 55 mg/m^2, at which 2 out of 3 dogs experienced grade 4 neutropenia.

Cohort Expansion and Outcome

Having established the MTD, an additional 20 dogs were enrolled into the first phase of the clinical trial cohort expansion and given TOC at the previously described dosing schedule and lomustine at a target dosage of 50 mg/m^2 once every 3 weeks. Of the first 28 dogs treated with lomustine at 50 mg/m^2 combined with pulse-administered TOC, 13 dogs had an objective tumor response (10 PR, 3 CR). Because the number of dogs experiencing an objective tumor response was >11, 13 additional dogs were included in the dose expansion phase of the study. This resulted in a total of 41 dogs

Table 1. Patient demographics and tumor characteristics for dogs enrolled in the dose expansion phase (n = 41).

	Median (Range) or Frequency (%)
Age (years)	8.2 (3.0–13.0)
Weight (kg)	28.2 (6.0–57.3)
Sex	
MC	19 (46.3)
M	3 (7.3)
FS	17 (41.5)
F	2 (4.9)
Breed	
Mixed breed	10 (24.4)
Boxer	10 (24.4)
Labrador retriever	7 (17.1)
Golden retriever	2 (4.9)
Pointer	2 (4.9)
Other (1 each)	10 (24.4)
Tumor dimension (cm)	6.7 (0.84*–21.8)
c-kit mutation status	
Positive	15 (36.6)
Negative	23 (56.1)
Not evaluable	3 (7.3)
Prior treatment	
Surgery	26 (63.4)
Chemotherapy	12 (29.3)
Radiation therapy	1 (2.4)
No prior treatment	2 (4.9)
Metastasis	
None	10 (24.4)
Local lymph node	26 (63.4)
Distant ± local node	5 (12.2)

*One dog initially had a tumor ≥1.0 cm that decreased after 3 days of prednisone before 1st cycle.

Table 2. Dose escalation cohorts.

Cohort	Toceranib Dose (mg/kg)[a]	Lomustine Dose (mg/m^2)	Dogs Treated	No. of Dogs Experiencing DLT
1	2.75	50	8[b]	1[c]
2	2.75	60	2	2[c]
3	2.75	55	3	2[c]

DLT, Dose-limiting toxicity.
[a]Toceranib administered days 1, 3 and 5 of each cycle.
[b]Two dogs were not evaluable for DLT assessment.
[c]All dose-limiting adverse events were grade 4 neutropenia.

evaluable for outcome evaluation at the MTD, because 1 dog was lost to follow-up 1 day after study commencement. The median administered dosage of lomustine was 47.6 mg/m^2 (range 30.3–54.5 mg/m^2) for a median of 3 cycles (range 1–15 mg/m^2). The median administered dosage of TOC was 2.65 mg/kg (range 2.5–2.85 mg/kg). Hematologic adverse events were most common with 34 dogs (82.9%) developing some degree of neutropenia 1 week post-lomustine. Eight dogs (19.5%) experienced grade 1 neutropenia, 10 (24.4%) developed grade 2 neutropenia, 7 (17.1%) developed grade 3 neutropenia and 9 (22%) experienced grade 4 neutropenia. Dose reductions were performed for 9 (22%) dogs experiencing neutropenia and no dose delays were performed for management of neutropenia. One dog developed grade 4 anemia which occurred at the time visceral disease progression was noted. Hepatotoxicity also was common, with 24 dogs (59%) developing increases in ALT activity; 9 dogs had severe increases (8 grade 3 and 1 grade 4) in ALT activity. Twelve (50%) dogs had increased ALT activity after 1 dose of lomustine and these increases generally were mild (8 grade 1, 4 grade 2). Seven (29%) dogs developed increased ALT activity after 2 doses, 2 (8%) after 3 doses, and 1 (4%) developed increased ALT activity after 5 doses of lomustine. Increased ALT activity was associated with a lomustine dose decrease (grade 3, n = 1) or dose delay (grade 1, n = 1; grade 2, n = 1; grade 3, n = 5). In addition, 1 dog had grade 4 increased ALT activity and was removed from the study. Hepatoprotectants were not administered prophylactically in this study but were used in the management of hepatotoxicity, with 13 dogs receiving Denamarin®[g] after increases in ALT activity were identified. Adverse events are summarized in Table 3. In summary, dose reductions of lomustine, treatment delays or both were performed for 16 dogs (39%) to manage adverse events in this study; these were implemented at the discretion of the supervising clinician. Seven dogs (17.1%) had a dose reduction of lomustine, 6 dogs (14.6%) had at least 1 treatment cycle delay, and 3 dogs (7.3%) had both a dose reduction of lomustine and a treatment delay performed. No dogs required dose reductions or delays in TOC administration.

Fifteen dogs (36.6%) experienced PR and 4 dogs (9.8%) experienced CR after lomustine and pulse-dose TOC for an ORR of 46%. Of the remaining dogs, 6 had SD, 15 developed PD, and 1 dog was not evaluable for response because of euthanasia by the primary care veterinarian before response assessment. The presence of a *c-kit* ITD was not associated with response to treatment ($P = 0.51$). The median TTMR was 21 days (range 7–175 days). The median PFS was 53 days

Table 3. Summary of adverse events (AE) occurring in the dose expansion phase of the study (n = 40 dogs) as defined by Veterinary Co-operative Oncology Group – Common Terminology Criteria for Adverse Events v1.0 (2004).

Adverse Event Category	Term	Grade 1	2	3	4
Hematologic	Neutropenia	8	10	7	9
	Anemia	8	2	1	1
	Thrombocytopenia	3	1		
GI	Vomiting	13	2		
	Diarrhea	7	4		
	Anorexia	6	2		
Hepatic	ALT elevation	7	8	8	1
Constitutional	Lethargy	1	4		

GI, Gastrointestinal; ALT, alanine aminotransferase.

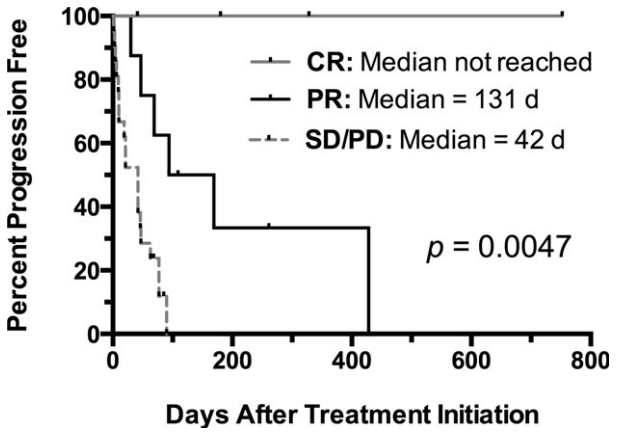

Fig 1. Kaplan–Meier curve depicting progression-free survival of dogs whose best response to treatment was either complete response (CR), partial response (PR), or stable disease (SD)/progressive disease (PD). *P* value indicates logrank test for trend across the 3 groups.

(range 1 to >752 days) and the median OS was 131 days (range 4 to >752 days).

The median follow-up time in censored patients was 235 days. On univariate analysis, variables associated with prolonged PFS included response to treatment (Fig 1), absence of metastasis, no previous chemotherapy, and smaller tumor diameter (<6.7 cm; Table 4). The median PFS was not reached for dogs experiencing CR, was 131.5 days for dogs experiencing PR, and was 77 days for dogs experiencing SD as their best response. Variables associated with increased OS on univariate analysis included no previous chemotherapy and smaller tumor diameter (Table 5). The presence of a *c-kit* activating mutation was not associated with outcome ($P = 0.99$ for PFS, $P = 0.92$ for OS). Variables that remained significant upon multivariate analysis for improved PFS included response, metastasis, and no prior chemotherapy. Response to treatment, small tumor diameter, and no prior treatment were associated with a significantly improved OS (Table 6).

Table 4. Factors evaluated for effects on progression-free survival.

	Groups	n	Median PFS	Logrank *P*	Logrank HR (95% CI)
Response	CR/PR	19	169		
	SD/PD	21	42	<0.0001	3.496 (2.6–11.88)
Delay/	Yes	16	73		
Reduction	No	25	47	0.0653	1.899 (0.9875–4.053)
Metastasis	Yes	31	47		
	No	10	104	0.0063	2.804 (1.396–5.366)
Previous	Yes	12	42		
Chemotherapy	No	29	77	0.0049	2.63 (1.586–10.21)
Tumor	≥6.7 cm	21	46.5		
Diameter	<6.7 cm	20	77	0.0147	2.143 (1.219–4.529)

PFS, Progression-free survival; HR, Hazard ratio; 95% CI, 95% confidence interval; CR, Complete response; PR, Partial response; SD, Stable disease; PD, Progressive disease.

Table 5. Factors evaluated for effects on overall survival.

	Groups	n	Median ST	Logrank *P*	Logrank HR (95% CI)
Response	CR/PR	19	168		
	SD/PD	21	118	0.4962	1.36 (0.5795–3.155)
Delay/	Yes	16	222		
Reduction	No	25	114	0.051	2.215 (1.002–4.537)
Metastasis	Yes	31	114		
	No	10	361	0.0759	2.182 (0.9962–4.947)
Previous	Yes	12	50		
Chemotherapy	No	29	146	0.0453	2.04 (1.04–5.798)
Tumor	≥6.7 cm	21	89		
Diameter	<6.7 cm	20	264	0.0195	2.162 (1.201–5.034)

ST, Survival time; HR, Hazard ratio; 95% CI, 95% confidence interval; CR, Complete response; PR, Partial response; SD, Stable disease; PD, Progressive disease.

Discussion

Lomustine at 50 mg/m^2 combined with pulse-administered TOC at 2.75 mg/kg can be safely administered to dogs with MCT with a DLT of neutropenia. A lomustine MTD of 50 mg/m^2 once every 3 weeks when combined with continuous dosing of TOC at 2.75 mg/kg every other day recently was reported when administered to dogs with tumors other than MCT; the DLT in this phase I study with continuous TOC administration also was neutropenia.[29] Other common adverse effects included hepatic and gastrointestinal toxicities, which were not unexpected based on the combination of drugs used in this protocol. Lomustine is known to cause hepatotoxicity in dogs, with reported rates of 83–86%.[30–32] Twenty-four dogs (59%) developed increases in ALT activity during this study, with 9 dogs having grade 3 or 4 increases. The incidence and severity of increased ALT activity was less than previously reported, which may be due in part to early intervention with hepatoprotectants, dose delays, or both when mild increases in ALT activity developed. The maximally tolerated dosage of lomustine in this protocol was 50 mg/m^2, which is 28.6–44% lower than lomustine dosages reported elsewhere for treatment of MCT.[7–9] This decreased lomustine dosage also may have decreased the frequency and severity of hepatotoxicity observed in this study as compared to previous studies. Dogs in this study also were receiving prednisone concurrently with lomustine and pulse-administered TOC, which routinely causes increases in alkaline phosphatase (ALP) activity and occasionally ALT as well.[33] Some of the increased ALT activity may have been due in part to prednisone administration. Because ALP activity was not routinely evaluated throughout the study, it is not possible to assess the influence of prednisone administration on ALT activity.

Table 6. Factors significant for progression-free survival (PFS) and overall survival (OS) on multivariable analysis.

PFS	*P*	HR	95% CI	OS	*P*	HR	95% CI
Response	<0.001	6.734	2.675–16.954	Response	0.001	5.451	1.922–15.46
Metastasis	0.024	0.286	0.096–0.851	Diameter	0.045	2.846	1.051–7.701
Previous Chemotherapy	0.002	0.245	0.101–0.593	Previous Chemotherapy	<0.001	0.166	0.061–0.453

HR, Hazard ratio; 95% CI, 95% confidence interval.

The adverse gastrointestinal effects observed in this study were mild to moderate and primarily occurred after the first dose of lomustine. This observation is in contrast to what has been reported for chronic TOC administration, during which up to 46% of dogs experienced some degree of gastrointestinal toxicity.[13] One potential mechanism of gastrointestinal toxicity secondary to TOC is the inhibition of the KIT protein on the interstitial cells of Cajal, resulting in gastrointestinal hypomotility. Pulse administration of TOC may have prevented long-term down-regulation of KIT signaling in the interstitial cells of Cajal, thereby decreasing gastrointestinal toxicity with this dosing schedule.

The ORR of 46% for pulse-administered TOC combined with lomustine is comparable to what previously has been reported for single-agent protocols,[5,7,12,13] but considerably higher than that reported with single-agent lomustine in a recent multicenter prospective trial (23%).[34] Although a randomized, prospective clinical trial would be required to determine if 1 protocol was superior to the other, combining pulse-administered TOC with lomustine may be appealing to some pet owners who do not want to pursue parenteral chemotherapy for their pet but also cannot financially commit to using TOC as monotherapy caused by the cost of the drug and associated monitoring. Not surprisingly, the presence of metastatic disease and lack of response to therapy were associated with a short PFS in multivariate analysis. Dogs that were previously untreated also had an improved PFS and OS as compared to dogs that had previously received chemotherapy for MCT. These findings suggest that although the combination of pulse-administered TOC and lomustine is efficacious, the combination may not overcome the MCT drug resistance that may have occurred in dogs that have failed other therapies. The presence of a *c-kit* ITD did not affect PFS or OS for this group of dogs, nor was it associated with response to treatment. This finding is in contrast to a previous publication evaluating TOC monotherapy for MCT in which *c-kit* mutation was associated with a higher likelihood of response,[13] and is in contrast to a study combining TOC and palliative radiation therapy, in which *c-kit* mutation was associated with an inferior outcome.[17] This finding highlights the importance of evaluating prognostic factors such as *c-kit* mutation status in a context-specific setting, rather than extrapolating from previous studies.

In conclusion, lomustine at a dosage of 50 mg/m^2 once every 3 weeks combined with pulse-dosed TOC was well tolerated, but the ORR was not superior to single-agent protocols. *c-kit* gene mutation status did not affect outcome. Notably, pulse administration of TOC was associated with a relatively low incidence of adverse gastrointestinal events, when compared with continuous exposure, and use of a lower dosage of lomustine may have contributed to a lower frequency of severe hepatotoxicity. A prospective, randomized trial evaluating whether this combination is advantageous over either lomustine or TOC alone should be considered based on these results.

Footnotes

[a] Qiagen DNEasy Blood and Tissue kit, Qiagen, Valencia, CA
[b] Phusion Blood Direct PCR kit, Thermo Scientific, Waltham, MA
[c] ABI Prism 3730xl genetic analyzer, Applied Biosystems, Carlsbad, CA
[d] GeneMarker v1.85, SoftGenetics, State College, PA
[e] Prism v. 6.0b, GraphPad Software, La Jolla, CA
[f] SPSS v. 21, IBM, Armonk, NY
[g] Nutramax Laboratories Veterinary Sciences, Inc., Lancaster, SC

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Conflict of Interest Declaration: Advisory board membership, consulting, speaker honoraria, travel and accommodations covered or reimbursed apply to D. Vail, D. Thamm and C. Clifford with regard to Zoetis, Inc. (formerly Pfizer Animal Health).

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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