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Combination of CCNU and DTIC Chemotherapy for Treatment of Resistant Lymphoma in Dogs

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Background: Pleotropic-glycoprotein (P-gp)–mediated resistance is the usual cause of relapse in dogs with lymphoma. 1-(2-chloroethyl)3-cyclohexyl-1-nitrosurea (CCNU) and 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC) are alkylating agents that are not affected by P-gp and lack cross-resistance to each other. A combination protocol offers the advantage of improved summation dose and synergistic activity.

Hypothesis: A combination of CCNU and DTIC that is well tolerated can be used to treat dogs with lymphoma that developed resistance or failed to respond to previously administered chemotherapy.

Animals: Fifty-seven dogs with lymphoma that were resistant to treatment with standard chemotherapy (L-CHOP; L-asparaginase, cyclophosphamide, doxorubicin, vincristine, prednisone).

Methods: Prospective phase I and II trials were performed. CCNU was given PO immediately before a 5-h IV infusion of DTIC. Concurrent antiemetics and prophylactic antibiotics were used. Treatments were administered every 4 weeks.

Results: Based on the results of 8 dogs in the phase I study, CCNU at 40 mg/m^2 PO combined with DTIC at 600 mg/m^2 IV was used to treat 57 dogs with resistant lymphoma. Thirteen (23%) dogs had a complete response (CR) for a median of 83 days and 7 (12%) had a partial response for a median of 25 days. The median L-CHOP CR duration of the dogs that did not respond to CCNU-DTIC was significantly longer than that of the dogs that did achieve remission with CCNU-DTIC (225 days versus 92 days, $P = .02$). The principal toxic event was neutropenia; the median neutrophil count 7 days after treatment was 1,275 cells/μL. Increases in alanine transaminase activity, possibly associated with hepatotoxicity, were detected in 7 dogs.

Conclusions and Clinical Importance: A combination of CCNU and DTIC can be an effective option to rescue dogs with resistant lymphoma.

Key words: Cancer; Canine; Dacarbazine; Lomustine; Relapse; Rescue; Summation dose; Tolerable-dose diagram.

Canine lymphoma is typically treated with cyclic chemotherapy consisting of alkylating agents (cyclophosphamide), antimicrotubule agents (vincristine), anthracyclines (doxorubicin), prednisone, and L-asparaginase (L-CHOP).[1,2] Complete response rates of 80% or greater are expected in dogs treated with L-CHOP–based protocols and, the median first remission duration is approximately 9 months.[1,3–7]

Rescue therapy attempts to establish remission in a patient who has failed first-line treatment or to reestablish remission in a patient who has relapsed after previous treatment. Resistance to antineoplastic drug therapy is the usual cause of relapse in dogs with lymphoma. Several mechanisms exist by which tumor cells acquire drug resistance. In dogs with lymphoma, overexpression of pleotropic-glycoprotein (P-gp) is one of the major factors leading to the multidrug resistance (MDR) phenotype.[8–10] Lymphoma cells with the MDR phenotype are resistant to several chemotherapeutics including antimicrotubule agents, anthracyclines, and prednisone—drugs commonly used to treat this disease. In contrast to antimicrotubule agents and anthracyclines, alkylating agents are not affected by the MDR phenotype. Additionally, different alkylating agents rarely have cross-resistance to each other.[11] These factors make them logical choices as rescue agents for dogs with lymphoma.

1-[2-chloroethyl]3-cyclohexyl-1-nitrosurea (CCNU, lomustine) is a bifunctional alkylating agent in the nitrosourea subclass. The initial step in the alkylation process is the transfer of a chloroethyl group from the chloroethyl-nitrosurea to the O^6 methyl group of guanine in DNA. Intra- and interstrand cross-links in DNA follow, resulting in inactivation of the DNA template, cessation of DNA synthesis, and, ultimately, cell death.[11] In dogs with resistant lymphoma, CCNU is an effective rescue treatment with an overall response rate of 27%.[12]

5-(3,3-Dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC, dacarbazine) is a nonclassical alkylating agent that acts via methylation of DNA, also at the O^6 methyl group of guanine.[11] There have been few controlled studies to evaluate the response of canine lymphoma to DTIC. A dog with cutaneous lymphoma and nodal involvement achieved a durable complete remission when treated with DTIC.[13] In 2 studies of dogs with relapsed lymphoma, complete and partial remissions were observed when DTIC was combined with doxorubicin.[14,15]

The prospective study reported here evaluates the combination of CCNU and DTIC to treat resistant canine lymphoma. Combination therapy offers possible advantages, including biochemical synergism, lack of cross-resistance (differential susceptibility of tumor cells to different agents), and higher achievable dose intensity, by exploiting nonoverlapping toxicities.[16,17] The objective of

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the phase I study reported here was to determine the maximal doses of CCNU and DTIC administered as combination therapy that could be tolerated in tumor-bearing dogs. The objectives of the phase II study were to evaluate the efficacy and toxicity of CCNU combined with DTIC every 4 weeks in dogs with lymphoma that had developed resistance or failed to respond to previously administered L-CHOP–based chemotherapy.

Materials and Methods

Study Subjects

From December 2004 to December 2006, client-owned dogs were evaluated for inclusion in this study. Dogs were considered eligible to receive combined treatment with CCNU and DTIC if they had confirmed multicentric lymphoma that had developed resistance or failed to respond to previously administered L-CHOP–based chemotherapy. Additional inclusion criteria included an expected survival of $\geq$14 days without treatment, body weight $\geq$5 kg, and adequate cardiac, renal, and hepatic function. Dogs with lymphoma of the skin, gastrointestinal tract, and nervous system and dogs that had received myelosuppressive chemotherapy within 14 days or had $\geq$grade 3 thrombocytopenia or $>$grade 1 gastrointestinal signs[18] (as depicted in Table 1) at baseline were excluded.

Initial Evaluation

Baseline evaluation included physical examination, a CBC with differential and platelet count, serum biochemical analysis, and urinalysis. Lymph nodes and organs were either directly measured with calipers or imaged and measured with radiographic or ultrasonographic methods. For each dog, the original immunophenotype and lymphoma stage and substage according to the World Health Organization staging system were recorded. Only dogs staged by use of full bloodwork, thoracic radiographs, abdominal ultrasound, and bone marrow cytology were considered to be originally staged. Dogs were not completely restaged before treatment with CCNU-DTIC.

CCNU-DTIC Administration

CCNU[a] was administered PO. CCNU is commercially available as 10, 40, and 100 mg capsules. In addition to the standard sizes, 5 mg reformulated capsules were prepared. The dose of CCNU was delivered to the nearest 5 mg. DTIC[b] was reconstituted in sterile water to achieve a concentration of 10 mg/mL, and the prescribed dose was further diluted in saline solution. The volume of saline used for dilution was based on body surface area (BSA) as follows: 1,000 mL saline for dogs $>1\ m^2$ BSA, 250 mL of saline for dogs 0.4–$1\ m^2$ BSA, and 100 mL saline for dogs $<0.4\ m^2$ BSA. Dolasetron[c] was administered as an antiemetic at a dosage of 0.6 mg/kg. DTIC and dolasetron were administered IV through an indwelling catheter. Specifically, dogs received an oral dose of CCNU, followed by a bolus of dolasetron, IV. The calculated dose of DTIC was then infused during a 5-h period. After treatment, dogs received a prophylactic antibiotic (trimethoprim–sulfadiazine,[d] 15 mg/kg PO q12h for 14 days) and a prophylactic antiemetic (metoclopramide,[e] 0.5 mg/kg PO q8h for 7 days).

Assessment of Response and Toxicity

Dogs were evaluated by a physical examination and CBC 7, 14, 21, and 28 days after treatment. Serum biochemical analysis and urinalysis were repeated on day 28. Tumor response was determined at each examination by measuring lymph nodes, organs, or both as described previously. Response to therapy was categorized as follows: complete response (CR), the disappearance of all clinical evidence of disease, and partial response (PR, $>$50% reduction but $<$100% reduction in the size of all measurable lesions). Response categories were required to persist for 21 days or more. Any response $<$PR or durations $<$21 days were defined as no response. Dogs that died before their first reevaluation were considered nonresponders.

Evidence of toxic effects of CCNU-DTIC was monitored by evaluation of the medical histories obtained from owners and results of physical examination and clinicopathologic data. Toxic effects were graded in accordance with predetermined criteria[18] (Table 1). If platelet clumping was detected on CBC, platelet counts were not

Table 1. Criteria used to grade[18] toxic effects in dogs administered the combination of CCNU (1-[2-chloroethyl]3-cyclohexyl-1-nitrosurea) and DTIC (5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide).

Toxic Effect and Grade	Signs
Neutropenia	
0	None
1	1,500–2,500 neutrophils/μL
2	1,000–1,499 neutrophils/μL
3	500–999 neutrophils/μL
4	$<$500 neutrophils/μL
Thrombocytopenia	
0	None
1	100,000–200,000 platelets/μL
2	50,000–99,000 platelets/μL
3	15,000–49,000 platelets/μL
4	$<$15,000 platelets/μL
Renal	
0	None
1	Serum creatinine concentration ULN–1.5$\times$ ULN
2	Serum creatinine concentration $>$1.5–2$\times$ ULN
3	Serum creatinine concentration $>$2–3$\times$ ULN
4	Serum creatinine concentration $>$3$\times$ ULN
Hepatic	
0	None
1	ALT ULN–1.5$\times$ ULN
2	ALT $>$1.5–3$\times$ ULN
3[a]	ALT $>$3–10$\times$ ULN
4	ALT $>$10$\times$ ULN
Anorexia	
0	None
1	Inappetance/coaxing, diet change required
2	Anorexia $<$3 days duration, no weight loss
3	Anorexia 3–5 days duration, weight loss
4	Anorexia $>$5 days duration, life-threatening
Vomiting	
0	None
1	$<$3 episodes in 24 hour
2	3–5 episodes in 24 hour; $<$3 episodes/d $<$5 days
3	$>$5 episodes in 24 hour; $>$4 days; fluid therapy
4	Life-threatening (hemodynamic collapse)
Diarrhea	
0	None
1	$>$2 stools/d over baseline
2	2–6 stools/d over baseline, IV fluids indicated $<$24 hour, not interfering with daily life
3	$>$6 stools/d over baseline, IV fluids $>$24 hour, hospitalization, interfering with daily life
4	Life-threatening (hemodynamic collapse)

[a] The original published grading system defines grade 3 as ALT $>$2$\times$ upper limit of normal and therefore was modified for use in the present study.

ULN, upper limit of normal; ALT, alanine transaminase.

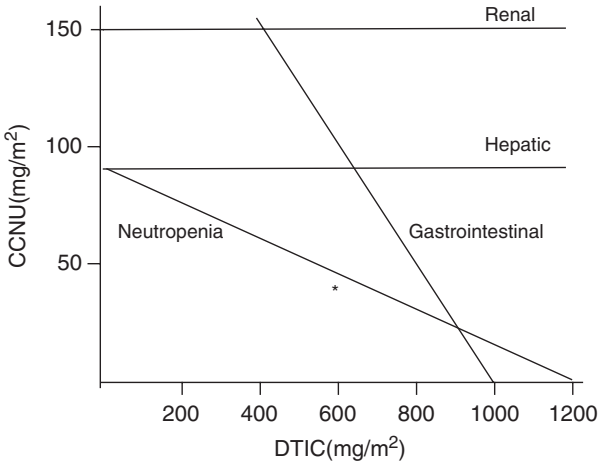

Fig 1. Tolerable-dose diagram illustrating the organ-specific dose-limiting toxicity (DLT) curves for various combinations of 1-(2-chloroethyl)3-cyclohexyl-1-nitrosurea (CCNU) and 5-(3,3-dimethyl -1-triazeno)-imidazole-4-carboxamide (DTIC). DLTs of DTIC were based on retrospective review of cases treated at our institution. DLTs of CCNU were based on previously published reports.[12,19] Hepatic and renal toxicity for CCNU are generally associated with cumulative dosing. Thrombocytopenia might be a cumulative toxicity in some dogs treated with CCNU but has been poorly described in the literature and therefore is not depicted. The dose combination for the protocol under investigation is indicated by the asterisk (*).

used for assessment of toxicity. Dose-limiting toxicity (DLT) for the CCNU-DTIC combination protocol was defined as grade 4 neutropenia, thrombocytopenia, or gastrointestinal toxicosis; $\geq$grade 2 renal toxicosis; or $\geq$grade 3 hepatic toxicosis.

Phase I Dose Escalation

This study was conducted as an open-label, phase I trial. Each dog was administered a single treatment of CCNU-DTIC. A tolerable-dose diagram illustrating the organ-specific DLTs for various combinations of CCNU and DTIC was prepared (Fig 1). DLTs of CCNU were based on previously published reports.[12,19] DLTs of DTIC were based on retrospective review of dogs treated with DTIC as a single agent at our institution. Starting dosages were selected by examining the relationship of the DLTs of both drugs. Based on this analysis, the starting dosages of CCNU and DTIC were set at 50 and 600 mg/m^2 BSA, respectively. Dose escalation was planned for CCNU in increments of 10 mg/m^2. The dosage of DTIC was to be held constant. Each dosage of CCNU-DTIC was to be administered to 3 dogs, provided that none of the treated dogs had DLT. If 1 of the 3 dogs in a group experienced DLT, 3 additional dogs would receive CCNU-DTIC at those same dosages. If no DLT was observed in the additional 3 dogs, the dosage of CCNU was escalated. If 2 or more dogs in a group had DLT, at least 3 additional dogs were to receive the preceding treatment dosage. The maximally tolerated dosage was defined as the dosage at which $\geq$2 of 3 or $\geq$2 of 6 dogs experienced DLT during their 1st cycle of treatment. The dosages recommended for the phase II trial of CCNU-DTIC were defined as the highest dosages at which $<$1 of 6 dogs experienced DLT.

Phase II Study for Resistant Canine Lymphoma

Dogs with resistant lymphoma were treated with CCNU-DTIC at dosages recommended in the phase I study. Treatments were administered every 28 days, as long as patients sustained a response (CR or PR) and had sufficiently recovered from any toxic effects associated with the preceding treatment. Evidence of drug toxicity was monitored as described previously. For dogs receiving more than 1 treatment, serum biochemistry and urinalysis were repeated every 4 weeks. All toxicoses were graded in accordance with predetermined criteria[18] (Table 1). For any dogs with a grade 4 hematologic toxicity, subsequent CCNU dosages were reduced by 10 mg/m^2. For any dogs with a $\geq$grade 3 gastrointestinal toxicity, subsequent dosages of DTIC were reduced by 100 mg/m^2. For dogs with pretreatment alanine transaminase (ALT) activity within the normal reference range (25–106 U/L), hepatotoxicity was suspected if ALT increased $\geq 3\times$the upper limit of normal (Table 1, grade 3 hepatotoxicity), and CCNU-DTIC was discontinued. For dogs with increased ALT before treatment, hepatotoxicity was suspected if ALT increased $\geq 2\times$the baseline value, and CCNU-DTIC was discontinued. CCNU-DTIC was discontinued if a dog developed $\geq$grade 2 renal toxicosis.

Statistical Analysis

All eligible dogs that began treatment with CCNU-DTIC were considered for use in estimating response and toxicity. Dogs that received treatment and died or were euthanized because of toxicity were still evaluated. The overall response rate was defined as the number of dogs achieving CR or PR, compared with the total number of dogs treated. CR and PR rates were defined as the number of dogs achieving CR or PR, respectively, compared with the total number of dogs treated. Response duration was calculated using the Kaplan-Meier method and was defined as the number of days from the 1st day of the CCNU-DTIC protocol until relapse for dogs that achieved CR or progression of disease for dogs that achieved PR. Dogs still in remission or lost to follow-up were included in analyses until the last day follow-up information was collected and were then censored. In addition, if treatment with CCNU-DTIC was discontinued because of toxicity for a dog in remission, the dog was censored at the time any alternative rescue therapies were started. The 95% confidence intervals (CI) were determined for response proportion and response duration. Overall survival was not evaluated because of confounding influences of euthanasia and the owner's willingness to pursue other treatments.

Hematologic toxic effects were summarized by summary statistics, and hematologic nadirs were reported as a minimum value for each dog and each treatment. Nonhematologic toxic effects were summarized as a maximum grade for a specific type of event for each treatment.

Responders (CR and PR) were compared with nonresponders with respect to weight, response to standard L-CHOP chemotherapy (CR versus PR or no response), overall L-CHOP CR duration (from start of L-CHOP chemotherapy to initiation of 1st rescue protocol), and CR with any previous protocol, including L-CHOP and prior rescue therapies. Factors examined for their potential influence on risk of developing grade 4 neutropenia included weight and overall L-CHOP CR duration. Factors examined for their potential influence on risk of developing hepatotoxicity included weight, overall L-CHOP CR duration, total number of treatments with CCNU, cumulative dose of CCNU per m^2 BSA, and pretreatment increase in ALT activity (normal versus increased, reference range 25–106 U/L) before receiving CCNU-DTIC. Student's *t*-tests were used for analysis of continuous Gaussian data and Mann-Whitney *U*-tests were used for continuous non-Gaussian data. Fisher exact test was used to analyze categorical data. All analyses were two-sided, and $P \leq .05$ was considered to be significant. All statistical calculations were performed using a computer software program.[f]

Results

Phase I Dose Escalation Study

Based on analysis of a tolerable dose diagram, starting dosages of CCNU and DTIC were 50 and 600 mg/m^2, respectively. Only 2 dogs were treated at these dosages, and both experienced grade 4 hematologic toxicity. The first became severely neutropenic (200 cells/μL) and thrombocytopenic (42,000 cells/μL) and developed a fever; it recovered after receiving supportive treatment consisting of IV fluids and antimicrobials. The other dog became severely neutropenic (300 cells/μL) but remained asymptomatic. The dosage of CCNU was subsequently reduced to 40 mg/m^2 and the dosage of DTIC was held constant at 600 mg/m^2. Six dogs were treated at these dosages, 1 of which experienced DLT in the form of grade 4 neutropenia. Based on these results, the maximum tolerated dosage combination was CCNU at 50 mg/m^2 combined with DTIC at 600 mg/m^2. The dosage combination to be used in the phase II evaluation was CCNU at 40 mg/m^2 and DTIC at 600 mg/m^2.

Phase II Study for Resistant Canine Lymphoma

Study Subjects. Fifty-seven dogs with resistant lymphoma were entered into the phase II study. Thirty-six were males (31 castrated) and 21 were females (19 spayed). Purebred dogs represented by 24 different breeds accounted for 67% (38 of 57) of the dogs. The remaining 33% (19 of 57) were mixed-breed dogs. The median body weight was 29 kg (range, 5–96 kg), and the median age was 8 years (range, 3–13 years). Of 34 dogs with available original staging information, 23 were stage V, 8 were stage IV, and 3 were stage III. The remaining 23 dogs were incompletely staged but the WHO stage in these dogs was at least stage III. Of 51 dogs with available original substage information, 23 were substage a and 28 were substage b. Thirty dogs had immunophenotyping performed when originally diagnosed; 15 were B-cell and 15 were T-cell. At baseline, 42 dogs had ALT activity within the reference range, and 15 dogs had increased ALT activity (median ALT, 128 U/L; range, 107–552 U/L).

Previous Treatments. Before treatment with CCNU-DTIC, all dogs received a median of 6 drugs (range, 5–9). In 54 dogs, initial treatment included an L-CHOP–based chemotherapy protocol, similar to that described by Garrett et al.[1] Three dogs were treated with cyclophosphamide, vincristine, and corticosteroids, followed by treatment with single-agent doxorubicin at the time of relapse. In addition to L-CHOP drugs, some dogs received other agents in their original protocol; 18 dogs received methotrexate, 18 received vinblastine, and 1 dog each received mitoxantrone, chlorambucil, and etoposide. Seven dogs were treated with half-body radiation therapy as part of their initial protocol. Forty-six dogs (81%) achieved CR to their original protocol. The remaining 11 dogs achieved PR or did not respond. The median overall 1st remission duration after the initial treatment protocol was 144 days (range, 35–481 days). All 57 dogs were considered resistant to first-line chemotherapy before treatment with CCNU-DTIC.

CCNU-DTIC was the first rescue protocol for 32 dogs and 25 received different rescue protocols before CCNU-DTIC (mechlorethamine, vincristine, prednisone, procarbazine, n = 14; mechlorethamine, vinblastine, prednisone, procarbazine, n = 10; single-agent etoposide, n = 1). No dog received CCNU or DTIC before entering the study. The overall median duration of time from 1st diagnosis of lymphoma to treatment with CCNU-DTIC was 158 days (range, 33–1,522 days).

CCNU-DTIC Treatments and Toxicoses

Ninety-eight CCNU-DTIC treatments were administered to the 57 dogs. The median number of treatments was 1 (range, 1–6). The number of treatments given to the dogs was as follows: 1 (n = 39), 2 (n = 7), 3 (n = 5), 4 (n = 1), 5 (n = 3), 6 (n = 2). After the 1st treatment, the dosage of CCNU was reduced from 40 to 30 mg/m^2 in 3 dogs because of neutropenia. Reduction of the CCNU dosage successfully avoided grade 4 neutropenia in one of these dogs. The other 2 dogs were euthanized because of progressive disease after dose reduction and CBC information was not available. No dogs experienced adverse gastrointestinal effects requiring dose reduction of DTIC.

Data on the toxic effects for dogs in the study are summarized in Table 2. The toxicoses represent the maximum grade of toxicoses observed for a specific dog after treatment. Neutropenia was the principal toxic effect. After the 1st treatment, CBCs were evaluated weekly and 11 of 42 (26%) dogs with available information developed grade 4 neutropenia (< 500 cells/μL). The median neutrophil nadir in these 42 dogs was 1,275 cells/μL and occurred on day 7 in all dogs. Neutrophil counts returned to the reference range 2–14 days after the nadir (median, 7 days). One of the 11 dogs that developed grade 4 neutropenia had received half-body radiation therapy as part of its initial protocol; the remaining 10 had received only chemotherapy. There was no difference between the dogs that developed grade 4 neutropenia and the dogs that did not with regard to body weight ($P = .40$) or overall 1st L-CHOP CR duration ($P = .77$).

Table 2. Treatment-related adverse effects after administration of CCNU-DTIC to dogs with resistant lymphoma.[a]

Toxic Effect	Number of Available	Grade 0	1	2	3	4
Neutropenia	42	5	10	11	5	11
Thrombocytopenia	29	15	6	5	2	1
Anorexia	49	49	0	0	0	0
Vomiting	46	38	7	1	0	0
Diarrhea	48	48	0	0	0	0
Hepatotoxicity	31	22	1	1	5	2
Renal toxicity	34	31	3	0	0	0

[a]Dogs received 1–6 treatments with CCNU-DTIC; 98 CCNU-DTIC treatments were administered to the 57 dogs. Results are expressed as the maximum grade of toxic effect observed for each dog.

CCNU, 1-[2-chloroethyl]3-cyclohexyl-1-nitrosurea; DTIC, 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide.

Four of 53 dogs (8%) with available information developed fever (range, 103.2–105.3°F) after treatment. In all dogs, fever was noted after the 1st treatment, between days 6 and 10. All 4 dogs were neutropenic (2 grade 4, 1 grade 3, 1 grade 1) and recovered with supportive treatment consisting of IV fluid administration and antibiotics.

Weekly platelet counts were available for 29 dogs. Weekly CBCs were performed in 42 dogs; however, platelet clumping precluded toxicity evaluation in 13 dogs. The median platelet nadir in the 29 dogs was 185,000 cells/μL (range, 12,000–401,000 cells/μL) and occurred at a median of day 14 (range, 7–28 days). Platelet counts returned to the reference range 7–21 days after the nadir (median, 7 days). One episode of grade 4 thrombocytopenia (<15,000 cells/μL) was detected on day 21 after treatment, when progressive lymphoma was also diagnosed. This dog had a history of thrombocytopenia when its lymphoma was out of remission.

Adverse gastrointestinal effects were uncommon and are summarized in Table 2. Eleven of 51 dogs (22%) with available information experienced mild adverse gastrointestinal effects. Two dogs vomited once during the DTIC infusion. Both of these dogs had been premedicated with ondansetron[g] (0.1 mg/kg IV) instead of dolasetron because dolasetron was unavailable. Four dogs had grade 1 vomiting on days 1 (n = 2), 2 (n = 1), or 10 (n = 1) after the infusion. No dogs experienced anorexia or diarrhea associated with DTIC.

Repeated serum biochemistry was available every 4 weeks during treatment. No dog experienced renal toxicity. Of 34 dogs with at least 1 repeated serum biochemistry, 7 (21%) developed hematologic changes suggestive of hepatotoxicity. The number of CCNU-DTIC treatments administered to these dogs before detection of biochemistry panel abnormalities ranged from 1 to 3 (median, 2 treatments) and the cumulative dosage of CCNU ranged from 40 to 120 mg/m^2 (median, 80 mg/m^2). Before CCNU-DTIC treatments, ALT activity was normal in 5 of the 7 dogs; 2 dogs had an increased ALT activity (128 and 201 U/L, respectively, reference range 25–106 U/L). At the time of suspected hepatotoxicity, the median ALT activity was 661 U/L (range, 347–6,551 U/L). Hyperbilirubinemia was detected in 1 dog (total bilirubin 4.3 mg/dL, reference range 0–0.3 mg/dL). Preprandial and postprandial bile acid concentrations were evaluated in 2 dogs and were abnormally high in both (222/181 and 40/44 μM, respectively; preprandial reference range, 0–13 μM; postprandial reference range, 0–30 μM). Other biochemical changes in dogs with hepatotoxicity were abnormally high serum activity of alkaline phosphatase (median, 2,001 U/L; range, 239–5,339 U/L; reference range, 12–122 U/L), aspartate transaminase (median, 154 U/L; range, 34–918 U/L; reference range, 16–50 U/L), and hypercholesterolemia (median, 449 mg/dL; range, 345–743 mg/dL; reference range, 124–335 mg/dL). Serum albumin, blood urea nitrogen, and glucose concentrations were normal in all 7 dogs. Diagnostic imaging or hepatic biopsies to further characterize liver abnormalities were not routinely performed. Based on palpation and cytology of peripheral lymph nodes, 3 of the 7 dogs were in CR (2) or PR (1) at the time hepatotoxicity was diagnosed. In one of these dogs, increased serum ALT activity (560 U/L) returned to normal (63 U/L) in 1 month after CCNU-DTIC was discontinued. Serum ALT activity decreased, but did not return to normal, in the other 2 dogs (ALT decreased from 2,152 to 214 U/L when retested 6 weeks later in 1 dog and ALT decreased from 6,551 to 784 U/L when retested 4 weeks later in the other). Four of the 7 dogs had clinical evidence of progressive lymphoma when hepatotoxicity was diagnosed; all were euthanized due to progressive disease within 1 week to 1 month after evidence of hepatotoxicity was first detected. No dogs died because of hepatotoxicity. There was no difference between dogs that developed liver toxicity and dogs that did not with regard to L-CHOP CR duration ($P = .10$), body weight ($P = .93$), total number of treatments with CCNU ($P = .61$), cumulative dosage of CCNU ($P = .60$), or pretreatment ALT (normal versus increased, $P = 1.0$).

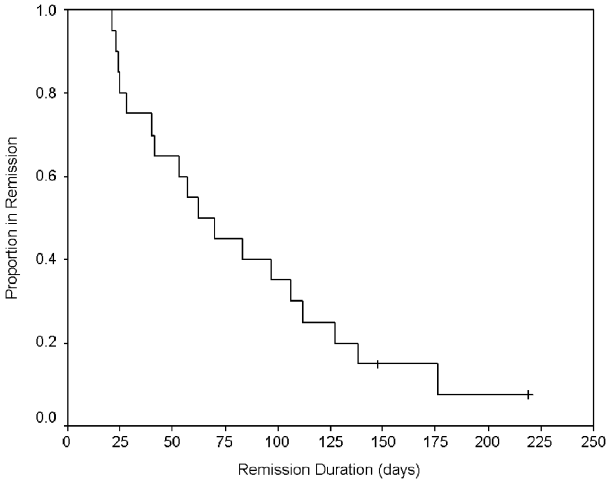

Fig 2. Kaplan-Meier curve depicting response duration for 20 of 57 dogs with resistant lymphoma treated with a combination of 1-(2-chloroethyl)3-cyclohexyl-1-nitrosurea (CCNU) and 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC). The overall median response duration was 62 days (95% CI, 34–90 days).

Response to Treatment. Response to treatment with CCNU-DTIC was evaluated in all 57 dogs. Eleven dogs were euthanized because of progressive lymphoma 2 to 20 days after treatment, and 1 dog was lost to follow-up 19 days after treatment. For purposes of the study, these dogs were considered nonresponders. The overall response rate was 35% (20 of 57; 95% CI, 24–48%) for a median duration of 62 days (95% CI, 34–90 days; range, 21–219 days; Fig 2). Thirteen dogs (23%) achieved a CR for a median duration of 83 days (95% CI, 31–135 days; range, 28–219 days). Seven dogs (12%) achieved a PR for a median duration of 25 days (95% CI, 0–71 days; range, 22–28 days; Fig 3). Phenotype was available for 12 of the 20 dogs that responded to CCNU-DTIC; 5 had B-cell lymphoma and 7 had T-cell lymphoma. Response to the CCNU-DTIC protocol based on immunophenotype and initial response to L-CHOP chemotherapy are depicted in Table 3. Ten dogs that responded to CCNU-DTIC

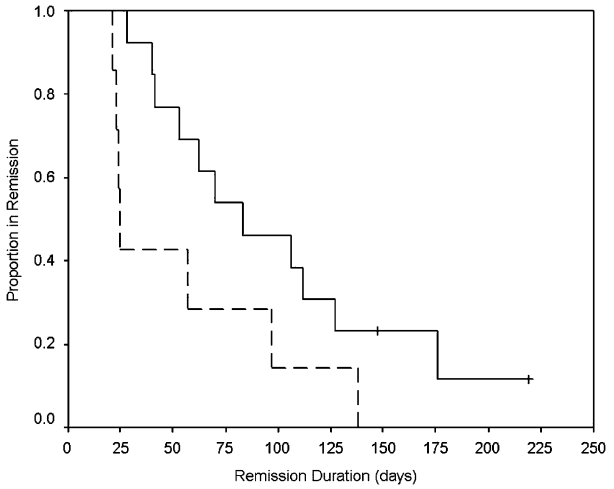

Fig 3. Kaplan-Meier curve depicting response duration for 13 dogs with resistant lymphoma that achieved a complete response (CR) to therapy (solid line) and 7 dogs that achieved partial response to therapy (broken line). The median duration of response for dogs achieving a CR to therapy was 83 days (95% confidence interval [CI], 31–135 days), and the median duration of response for dogs achieving partial response was 25 days (95% CI, 0–71 days).

had responses that were of a longer duration than their overall 1st L-CHOP remission duration. Five of these dogs had T-cell lymphoma and 5 had B-cell lymphoma. The first L-CHOP CR duration was significantly associated with response. The median L-CHOP CR duration of 30 nonresponders was significantly longer than that of 16 responders (225 days versus 92 days, $P = .02$). There was no significant difference between responders and nonresponders with respect to weight ($P = .23$), L-CHOP response (CR versus PR/no response, $P = .73$), or CR with any previous protocol ($P = .71$).

Discussion

The starting dosages for the phase I trial were selected based on a tolerable-dose diagram by means of neutropenia and gastroenteritis as possible dose-limiting toxicities. The first 2 dogs treated with the initial dosages experienced severe neutropenia. This outcome emphasizes that there are limitations associated with the use of tolerable-dose diagrams to predict toxicoses associated with combinations of chemotherapeutics, including poor approximation of organ-specific toxicities and unexpected synergistic adverse effects.[17] Dogs in the phase I study (and phase II study) were not restaged immediately before treatment (ie bone marrow aspiration cytology was not performed), and many were heavily pretreated with multiple drugs and protocols. Although unlikely, the degree of myelosuppression observed may have been confounded by both of these factors. Despite the high incidence of severe neutropenia in the phase II study, very few dogs developed signs of infection. Dogs received prophylactic antibiotics, but to date, no randomized studies in the veterinary literature reveal that prophylactic use of antibiotics with CCNU is beneficial.

Table 3. Response to the CCNU-DTIC combination protocol based on previous response to L-CHOP chemotherapy and immunophenotype.[a]

		Response to CCNU-DTIC		
	Number of Cases	CR	PR	NR
CR to L-CHOP				
Overall	46	10	5	31
B-cell	9	2	0	7
T-cell	13	4	2	7
PR/NR to L-CHOP				
Overall	11	3	1	7
B-cell	6	3	0	3
T-cell	2	0	1	1

[a]Immunophenotype information was available from 30 of the 57 dogs.

L-CHOP, L-asparaginase, cyclophosphamide, doxorubicin, vincristine, prednisone; CR, complete response; PR, partial response; NR, no response; CCNU, 1-[2-chloroethyl]3-cyclohexyl-1-nitrosurea; DTIC, 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide.

The dosages used in the phase II study reported here are considerably lower than those of CCNU and DTIC when used as single agents. As a single agent, CCNU at 90 mg/m^2 has been revealed to be effective for dogs with relapsed lymphoma.[12] Response information for dogs treated with single-agent DTIC is not available; however, a dosage of 800 mg/m^2 has been suggested for use.[20] In a wide variety of clinical situations, there are many potential advantages of using 2 drugs simultaneously instead of alternating.[21] Summation dose is a mathematical model that calculates the sum of the fractional doses for multiple drugs in a combination protocol and provides a means to make comparisons among protocols.[16,22] Based on the previously mentioned single-agent dosages, the fractional doses of CCNU and DTIC in our phase II study were 0.44 (40/90) and 0.75 (600/800), respectively. The summation dose, therefore, was 1.19. Assuming CCNU and DTIC are equally efficacious for treating relapsed lymphoma, each drug when used individually would have a relative dose of 1.0; therefore, the intensity of the combination protocol exceeds that of single-agent protocols, suggesting that despite lower individual dosages, the combination might lead to superior activity.

The cellular target leading to antitumor activity for both DTIC and CCNU is the O^6 methyl group of guanine in DNA. Cellular resistance most commonly occurs because of increased expression of O^6-alkylguanine DNA alkyltransferase (O^6-AGT), the predominant enzyme responsible for repair of alkylated DNA.[23] Despite a common target and mechanism of resistance, alkylating agents like DTIC and CCNU still may not have cross-resistance.[11] In fact, biochemical synergism has been reported with the combination of DTIC and nitrosoureas like CCNU.[11] Nitrosoureas deplete O^6-AGT, leading to sensitization of cells to the methylating activity of DTIC.[11] Lack of cross-resistance and synergistic activity are compelling arguments for combining CCNU and DTIC to treat dogs with resistant lymphoma, but it is possible that either agent alone was the drug that caused the responses seen in this study.

The overall response rate (35%; 95% CI, 24–48%) and response duration (62 days, 95% CI, 34–90 days) suggests that CCNU-DTIC might not be significantly different from other CCNU- or DTIC-containing protocols reported for dogs with resistant or relapsed lymphoma. For example, in 1 study, 8 of 15 dogs (53%) responded to DTIC combined with doxorubicin, but the duration of response was not reported.[15] Single-agent CCNU was evaluated in 41 dogs with resistant lymphoma, and 11 dogs (27%) achieved a complete or partial remission for a median of 86 days.[12] The combination of CCNU, vincristine, procarbazine, and prednisone was examined in 44 dogs with relapsed lymphoma; 52% responded for a median of 106 days.[24] However, the authors recommended the protocol be revised because 9% of the dogs died of treatment-related complications and 30% developed signs consistent with sepsis.[24] Finally, 87% of dogs with relapsed lymphoma responded to the combination of CCNU, L-asparaginase, and prednisone for a median of 63 days; however, only 24 of the 31 treated dogs were considered to have failed L-CHOP.[25] Direct comparisons between the results reported here and those of other rescue protocols are problematic; sample size, patient demographics, and adherence to intention-to-treat criteria differed among studies and most important, at the time of rescue therapy, dogs in all studies were not necessarily resistant to L-CHOP drugs. A prospective evaluation of the different protocols would be necessary to determine the optimal use of CCNU and DTIC to treat dogs with lymphoma.

Whether or not dogs responded to the CCNU-DTIC protocol was associated with the duration of time they were in CR from their initial L-CHOP chemotherapy protocol. The median L-CHOP duration of CR of dogs that did not respond to CCNU-DTIC was significantly longer than that of dogs that did respond. The Goldie-Coldman hypothesis predicts that random mutations occur within a tumor cell population that confer cytotoxic resistance.[23] This is evidenced by the fact that at the time of relapse, dogs with lymphoma have increased expression of P-gp compared with pretreatment.[9,10] Although P-gp does not mediate resistance to alkylating agents, likely other less well-characterized mechanisms are upregulated as well. It is possible that dogs did not respond to CCNU-DTIC after a lengthy L-CHOP protocol because residual lymphoma cells underwent mutations conferring mechanisms of resistance such as glutathione-*S*-transferase[26] or O^6-AGT. Future studies should be carried out to correlate response to CCNU-DTIC and the various forms of drug resistance.

The T-cell phenotype in canine lymphoma is generally associated with a poor response to chemotherapy.[2] Some authors have suggested that improved outcomes in dogs with T-cell lymphoma might be achieved by including alkylating agents early in treatment regimens.[27] Canine epitheliotropic lymphoma is an example of a T-cell lymphoma that responds poorly to most chemotherapeutics; however, CCNU appears to be a very effective treatment agent. In 2 combined studies of 82 dogs with cutaneous T-cell lymphoma, 80% experienced complete or partial remission after treatment with CCNU.[28,29] There is 1 case report of a dog with epitheliotropic lymphoma achieving complete remission after DTIC chemotherapy.[13] In the present study, we did not evaluate dogs with cutaneous lymphoma. However, at least 7 of the 20 dogs that experienced a remission when treated with the combination of CCNU and DTIC had multicentric T-cell lymphoma, and some of the remissions were for a longer duration than the 1st remission achieved with L-CHOP chemotherapy. It might be possible to improve the overall prognosis for dogs with T-cell lymphoma by including CCNU-DTIC as part of initial therapy, but more information about immunophenotype and response to the combination protocol is needed.

Abnormalities in liver enzyme activity might be an indication of CCNU-induced hepatotoxicity.[12,19,30] The combination protocol was discontinued in 7 dogs because of marked increases in serum ALT activity, representing 21% of 34 dogs with available repeated biochemistry results. Dogs in the study received prophylactic trimethoprim-sulfadiazene. Sulfonamide antimicrobials have been associated with dose-dependent hypersensitivity reactions, including acute hepatotoxicity.[31] Although unlikely, we cannot exclude the possibility that sulfonamide toxicity might have contributed to the increase in ALT activity seen in dogs in our study. The definition of hepatotoxicity in our study was based on our clinical experience using CCNU monotherapy in dogs with various tumors. This definition differs from the Veterinary Co-operative Oncology Group Common Terminology for Adverse Events.[18] Currently, there is no consensus regarding stopping requirements for CCNU administration in dogs.[28,29,32] In a large retrospective study, hepatotoxicity was documented in 11 of 179 (6%) dogs treated with CCNU. Criteria for hepatotoxicity in that study included clinical, serum biochemical, and histopathological changes identified after CCNU therapy.[30] In the present study, the CCNU-DTIC protocol was discontinued based on increases in ALT activity alone; only 3 of the 7 dogs had evidence of liver dysfunction (based on hyperbilirubinemia in 1 dog and increased postprandial bile acid concentrations in 2 dogs). Because definitions varied, direct comparisons between the incidence of liver toxicity in the study reported here and the frequency in the report by Kristal et al[30] cannot be made. However, the median number of CCNU doses and median total cumulative CCNU dose in dogs with suspected hepatic damage were lower than the values reported by Kristal et al[30] (2 doses, 80 mg/m^2 versus 4 doses, 350 mg/m^2). This observation might suggest an additive or synergistic toxicity when CCNU and DTIC are combined. A prospective, randomized study with a standardized schedule for monitoring liver function in dogs treated with CCNU alone and the combination of CCNU-DTIC will be needed to answer this question.

In conclusion, a protocol combining CCNU and DTIC offers improved summation dose and possibly lack of cross-resistance and synergistic activity. Results of the phase II study demonstrate that the protocol is an effective regimen to rescue dogs with lymphoma that fail to respond to or become refractory to previously administered L-CHOP chemotherapy.

Footnotes

[a] CeeNu, Bristol Laboratories, Princeton, NJ
[b] DTIC-Dome, Bayer Laboratories, West Haven, CT
[c] Anzemet, Aventis Pharmaceuticals Inc, Kansas City, MO
[d] Tribrissen, Interfarm, Auppauge, NY
[e] Reglan, Pliva, East Hanover, NJ
[f] SPSS 10, Statistical Analytical Software, Chicago, IL
[g] Zofran, Glaxo Smith Kline, Triangle Park, NC

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