

Assessment of GS-9219 in a Pet Dog Model of Non-Hodgkin's Lymphoma

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Abstract Purpose: To assess, in dogs with naturally occurring non-Hodgkin's lymphoma, pharmacokinetics, safety, and activity of GS-9219, a prodrug of the nucleotide analogue 9-(2-phosphonyl-methoxyethyl) guanine (PMEG), which delivers PMEG and its phosphorylated metabolites to lymphoid cells with preferential cytotoxicity in cells with a high proliferation index such as lymphoid malignancies.

Experimental Design: To generate proof-of-concept, a phase I/II trial was conducted in pet dogs ($n = 38$) with naturally occurring non-Hodgkin's lymphoma using different dose schedules of GS-9219. A subset of dogs was further evaluated with 3'-deoxy-3'-¹⁸F-fluorothymidine positron emission tomography/computed tomography imaging before and after treatment.

Results: The prodrug had a short plasma half-life but yielded high and prolonged intracellular levels of the cytotoxic metabolite PMEG diphosphate in peripheral blood mononuclear cells in the absence of detectable plasma PMEG. Dose-limiting toxicities were generally manageable and reversible and included dermatopathy, neutropenia, and gastrointestinal signs. Antitumor responses were observed in 79% of dogs and occurred in previously untreated dogs and dogs with chemotherapy-refractory non-Hodgkin's lymphoma. The median remission durations observed compare favorably with other monotherapies in dogs with non-Hodgkin's lymphoma. High 3'-deoxy-3'-¹⁸F-fluorothymidine uptake noted in lymphoid tissues before treatment decreased significantly after treatment ($P = 0.016$).

Conclusions: GS-9219 was generally well tolerated and showed significant activity against spontaneous non-Hodgkin's lymphoma as modeled in pet dogs and, as such, supports clinical evaluation in humans.

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Non-Hodgkin's lymphoma is the fifth leading cause of cancer deaths and the second fastest-growing form of cancer in the United States. In the latest compilation of Surveillance, Epidemiology and End Results reports, the incidence and death rate for non-Hodgkin's lymphoma continue to increase despite an overall decrease in overall cancer incidence (1). Therefore, there is still a major unmet medical need in non-Hodgkin's lymphoma patients for novel agents with improved efficacy compared with existing treatment modalities, especially in patients who have failed frontline therapy.

The acyclic nucleotide phosphonate 9-(2-phosphonyl-methoxyethyl) guanine (PMEG) forms an active phosphorylated metabolite, PMEG diphosphate, in cells and causes cytotoxicity in dividing cells due to potent inhibition of the nuclear DNA polymerases α , δ , and ϵ by causing DNA chain termination, resulting in inhibition of DNA synthesis (2). In rodent

Translational Relevance

Canine non-Hodgkin's lymphoma bears many similarities to human non-Hodgkin's lymphoma and is an attractive model for preclinical studies. There remains a major unmet medical need in non-Hodgkin's lymphoma patients for novel agents with improved efficacy compared with existing treatment modalities, especially in patients who have failed frontline therapy. We report here a proof-of-concept investigation of the novel nucleotide analogue pro-drug GS-2919 through the inclusion of pet dogs with spontaneously arising non-Hodgkin's lymphoma. This study provided valuable information before initiating human investigations: adverse events were generally transient, manageable, and dose and schedule dependent; the active form of the drug persisted selectively in peripheral blood mononuclear cells, prompting further evaluation of regimens using more frequent administration, and a favorable therapeutic index was shown in both treatment-naive and chemotherapy-refractory cases of non-Hodgkin's lymphoma in pet dogs. Thus, GS-9219 is a promising development candidate and phase I investigations of GS-9219 in human patients with lymphoid malignancies are in progress.

models, PMEG has activity against leukemia and melanoma (3). However, the utility of PMEG as an anticancer agent is limited by its poor cellular permeability and nonspecific toxicity, especially for the kidney and gastrointestinal tract (3–5). GS-9219, a novel double prodrug of PMEG, was designed as a cytotoxic agent that preferentially targets lymphoid cells (6). In lymphocytes, GS-9219 is hydrolyzed intracellularly to 9-(2-phosphonylmethoxyethyl)-*N*⁶-cyclopropyl-2,6-diaminopurine (cPrPMEDAP) and subsequently deaminated to PMEG. PMEG is then converted to its active phosphorylated form, PMEG diphosphate (6). The fact that rodents, unlike humans or dogs, have high plasma levels of carboxyesterase, which rapidly metabolizes GS-9219, precluded their use in

preclinical models (7). Evaluation of GS-9219 in normal beagle dogs previously showed, at doses that were generally well tolerated, selective depletion of replicative lymphoid tissues while sparing other tissues. Preliminary antineoplastic activity in dogs with naturally occurring non-Hodgkin's lymphoma has also been shown (6).

Canine non-Hodgkin's lymphoma has proven to be a relevant model for preclinical evaluation of new therapeutics for both initial induction and rescue of drug-resistant relapse (8–14). Evaluation of novel therapeutic approaches in dogs with spontaneous cancer offers potential benefit to canine patients and a rapid assessment of therapeutic index. Because the tumors arise spontaneously in an immunologically intact host and have greater heterogeneity than passaged cell lines, it is not surprising that responses to standard chemotherapeutic agents in canine malignancies are similar to those of the corresponding tumors in man and that the preclinical results attained may be more predictive of activity in humans (8, 15, 16). Non-Hodgkin's lymphoma in dogs represents a relatively homogenous population with respect to histologic type as defined by the REAL/WHO or National Cancer Institute-Working Formulation schema (85% are medium- to high-grade B-cell non-Hodgkin's lymphoma), with the majority being diffuse large B-cell lymphoma (17). As in people, non-Hodgkin's lymphoma in pet dogs is initially highly responsive to standard chemotherapy with ~90% of dogs achieving a complete remission with multidrug (e.g., CHOP) chemotherapy; however, cures are rare in dogs as relapse of drug-resistant disease occurs in the majority of cases (85%), with median survival durations of ~1 year following multidrug treatment protocols (16). To generate proof-of-concept, pharmacokinetic, safety, and activity data in lymphoid malignancies, a dose/schedule-finding and activity trial with GS-9219 monotherapy was initiated in pet dogs with naturally occurring chemotherapy-naive or chemotherapy-refractory advanced-stage non-Hodgkin's lymphoma.

Materials and Methods

Subject population. Pet owners presenting to the School of Veterinary Medicine, University of Wisconsin-Madison, or the Veterinary Medical Center, College of Veterinary Medicine & Biomedical Sciences, Colorado

Table 1. GS-9219 scheduled treatment regimens evaluated

Cohort	GS-9219 dose (mg/kg)*	Frequency [†]	Dogs treated	Dose-limiting adverse events	Best response observed
Cohort 1-low	0.20	Daily for 5 d, every 21 d	7 [‡]	1	5 CR/2 PR
Cohort 1-high	0.29	Daily for 5 d, every 21 d	6 [§]	3	5 CR/1 PR
Cohort 2-low	0.66	Once every 7 d	3	0	2 CR/0 PR
Cohort 2-high	0.82	Once every 7 d	6 [§]	2	3 CR/2 PR
Cohort 3-low	0.66	Once every 14 d	3	0	1 CR/0 PR
Cohort 3-high	0.82	Once every 14 d	6 [§]	1	3 CR/2 PR
Cohort 4-low	0.66	Once every 21 d	3	0	2 CR/0 PR
Cohort 4-high	0.82	Once every 21 d	4	0	2 CR/0 PR

*Based on GS-9219 free base.

[†]Each regimen was to be repeated for five total treatment cycles.

[‡]One dog was diagnosed with a second malignancy after entry and a seventh dog was added to cohort.

[§]Cohort expanded to 6 dogs due to a single dose-limiting adverse event in first 3 dogs.

^{||}One dog died of tumor-related complications before response evaluation and an additional dog was added to the cohort.

Table 2. Subject demographics

Characteristics	Value (%)
Age (y)	
Mean	7.5
Median	8
Range	2-14
Gender	
Male intact	1
Male neutered	22
Female intact	3
Female neutered	12
Tumor stage*	
III	12
IV	13
V	13
Tumor immunophenotype	
B cell	29
T cell	9
Prior Treatment	
Naive	17
Prior chemotherapy	21
CHOP combination	15
Doxorubicin monotherapy	4
CCNU/L-asparaginase/prednisone	1
Chlorambucil/prednisone	1

Abbreviations: CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone (16); CCNU (lomustine), L-asparaginase, prednisone rescue protocol (16).

*WHO staging for tumors in domestic animals (22).

State University, were offered entry into this study for treatment of their dogs with GS-9219 under compliance with the Animal Care and Use Committees of the University of Wisconsin-Madison and Colorado State University and the Clinical Review Board of the Colorado State University Veterinary Medical Center. All owners signed informed consent documents, including a statement to allow necropsy in the event of their animal's death, whenever possible. Dogs were evaluated by complete physical examination, routine clinical biochemistry with electrolytes, hematology and urinalysis, thoracic radiographs, bone marrow aspiration cytology, and diagnostic lymph node biopsy inclusive of histology and immunohistochemistry to confirm non-Hodgkin's lymphoma and determine immunophenotype. Concurrent antineoplastic therapy was not allowed. Previous cytotoxic chemotherapy was allowable, with at least a 3-week washout from the most recent treatment.

GS-9219 monotherapy protocols. GS-9219 was administered in all protocols by a 30 min intravenous infusion in 5% dextrose for injection, USP (2 mL/kg body weight). Four different schedules were evaluated, each with two dosing levels for a total of eight treatment cohorts (Table 1). Initial starting dose was chosen based on results from previous standard toxicology studies in beagle dogs (6). Several dosing schedules were initiated to evaluate potential treatment regimens. A standard 3 + 3 phase I cohort design was employed where 3 dogs were initiated in each cohort and observed for dose-limiting toxicities (DLT). A DLT was defined as persistent grade ≥ 3 for all adverse events, excluding neutropenia where a grade 4 adverse event was deemed dose-limiting. If no DLTs were observed in the first cohort of animals, a second cohort was treated on the same regimen at the higher dose. If a DLT was observed in 1 dog, the cohort was expanded to 6 dogs. If two or more DLTs were noted in any cohort, no further dose escalations were done.

Pharmacokinetic analysis of plasma and peripheral blood mononuclear cell levels of GS-9219 and its metabolites. A subset of dogs was evaluated for pharmacokinetics. Blood for plasma levels was collected at various time points into sodium fluoride potassium oxalate Vacutainer tubes (BD Biosciences) and centrifuged at 4°C to separate plasma. Plas-

ma was prepared by protein precipitation by adding acetonitrile and formic acid to a final concentration of 60% and 0.2%, respectively, in the presence of internal standards (a methylated version of PMEG and its corresponding phosphoramidate prodrug). Samples were dried in a MiVac Duo Concentrator (Genevac) and reconstituted in sterile water.

Blood for intracellular peripheral blood mononuclear cell (PBMC) levels was also collected 24 h post-dose into sodium citrate CPT Vacutainer tubes (BD Biosciences) for PBMC isolation. Following centrifugation through a thixotropic polyester gel and a Ficoll-Hypaque solution, isolated PBMCs were resuspended in normal saline. The cell suspension was centrifuged to pellet cells and isolated cells were resuspended in 70% methanol lysis buffer. Lysed samples were dried and resuspended at 1 million cells/10 μ L calf intestinal phosphatase buffer with internal standards and treated with 1 unit calf intestinal phosphatase at 37°C for 2 h (buffer and enzyme obtained from Sigma-Aldrich). Reactions were stopped by adjusting to 60% acetonitrile followed by drying and resuspension at 1 million cells/10 μ L in water.

All liquid chromatography-tandem mass spectrometry analyses used a HTS PAL autosampler (Leap Technologies) with cooled stacks, a Shimadzu LC-20AD ternary pump system (Shimadzu Scientific Instruments), and a Sciex API-4000 mass spectrometer (Applied Biosystems) operating in multiple-reaction monitoring and positive ionization modes. Liquid chromatography separation for the analysis of GS-9219, cPrPMEDAP, and PMEG was done using a Synergi Max RP-80 4 μ m 150 \times 2.0 mm column (Phenomenex) and a multistage linear gradient from 4% to 91% acetonitrile in 0.2% formic acid at a flow rate of 250 μ L/min. Standard curves and quality-control samples were used to ensure appropriate accuracy and precision. Noncompartmental pharmacokinetic analysis was done using WinNonLin version 5.0.1 (Pharsight).

Safety evaluation. Adverse events were graded according to the Veterinary Cooperative Oncology Group-Common Terminology Criteria for Adverse Events version 1.0 (18) modified from the National Cancer Institute-Common Terminology Criteria for Adverse Events used in humans. Toxicity evaluations were based on physical examination, client questionnaire, CBC, biochemistry profile, and urinalysis and were done pretreatment and 1 week post-treatment for all five cycles in the once every 7 days, once every 14 days, and once every 21 days cohorts. For dogs in cohort 1 (five consecutive daily treatments out of every 21 days), toxicity evaluations were done pretreatment and day 9 of every cycle. In dogs experiencing prolonged grade 3 adverse events, dose reductions or prolongation of the treatment interval was instituted. In the event of death or euthanasia, necropsy evaluation was done whenever possible.

Activity evaluation. Although assessment of lymph node size, computed tomography (CT), positron emission tomography (PET)/CT, and flow cytometric analysis is routine for determining standard response criteria for human patients with non-Hodgkin's lymphoma (19, 20), availability of advanced imaging limits similar assessments in veterinary patients. Clinical response in veterinary cancer patients with non-Hodgkin's lymphoma is generally based on summation of longest diameters of affected peripheral nodes, as measured with calipers, followed by the application of Response Evaluation Criteria in Solid Tumors of solid tumors for the pretreatment and post-treatment measurements (21). In the study reported here, response was assessed using a combination of lymph node size reduction (Response Evaluation Criteria in Solid Tumors), radiographic assessment of thoracic lesions, and cytologic assessment of previously affected nodes. Additionally, antitumor activity was further evaluated in a subset of 9 dogs by 3'-deoxy-3'-¹⁸F-fluorothymidine (FLT) PET/CT before and after treatment.

Antitumor activity was evaluated 7 days following the initial treatment with GS-9219, at the day of each subsequent treatment cycle, and monthly following completion of all GS-9219 treatments. A complete response (CR) was defined when post-treatment nodal size was deemed within normal limits (minimum of 75% reduction in the sums of the longest diameters) and was confirmed with cytologic assessment of a previously affected lymph node. A partial response (PR) was defined as a 30% decrease in the sums of the longest diameters of measurable involved nodes, and progressive disease was defined as a 20%

Table 3. Pharmacokinetic parameters following 30 min intravenous infusion of GS-9219 at 0.82 mg/kg

	Plasma				PBMC
	$t_{1/2}$ (h)	C_{max} (nmol/L)	T_{max} (h)	$AUC_{(0-\infty)}$ (nmol/L h)	Total C_{24} (nmol/L)*
PMEG	ND [†]	<12.3	ND	ND	4,020 ± 4,400
cPrPMEDAP	4.02 ± 0.30	404 ± 91	2.00 ± 0	3,700 ± 1,140	456 ± 736
GS-9219	0.33 ± 0.20	4,280 ± 1,550	0.32 ± 0.16	2,910 ± 830	NP

NOTE: Plasma and PBMC pharmacokinetic parameters are the mean ± SD based on $n = 8$ and 6 dogs, respectively. NP, not performed.

*Total concentration represents intracellular concentrations of parent nucleotide and all of its phosphorylated species measured following dephosphorylation.

[†]ND, not determined, as plasma levels of PMEG were below level of detection.

increase in the sums of the longest diameters of measurable involved nodes or newly arising lesions. Dogs that experienced progressive disease before completion of all GS-9219 cycles were removed from study and allowed to seek additional antitumor therapy at the discretion of the owner. Dogs that experienced relapse of their non-Hodgkin's lymphoma after completion of treatment with GS-9219 were offered either retreatment with GS-9219 monotherapy or other additional antitumor therapy at the discretion of the owner.

Median first remission duration (FRD) was defined as the time from documented attainment of remission until relapse (in the case of complete responders) or the documentation of progression (in those dogs achieving only a partial remission).

FLT-PET/CT. Nine dogs were evaluated by FLT-PET/CT before and after treatment based on scheduling and availability of scanner access. The FLT-PET/CT imaging studies were done on a clinical GE Discovery LS PET/CT scanner at University of Wisconsin hospitals and clinics. FLT was obtained from the cyclotron and radiopharmaceutical laboratory at the Department of Medical Physics at the University of Wisconsin-Madison. Approximately 6 mCi (220 MBq) of FLT activity was administered per scan. The whole-body FLT-PET/CT image acquisition with 10 min scan per bed position was initiated after 60 ± 10 min post-injection. All dogs were initially scanned in the preceding 24 h before their initial GS-9219 treatment and scheduled for subsequent scans 5 days (±1 day) following initial dose and 3 weeks following completion of five cycles of treatment ($n = 4$ dogs) or just before the fourth cycle of chemotherapy and 3 weeks following completion of five cycles of treatment ($n = 5$ dogs).

For each study, the mean maximum body mass standardized uptake values for FLT were calculated. Amira (Mercury Computer Systems) was used to identify regions of interest and perform data analysis.

Statistical analysis. Median FRD time for dogs in this study was determined from survival curves generated using the Kaplan-Meier method, which accounts for dogs that were alive, lost to follow-up, died from unrelated causes, or had not relapsed at the time of analysis. The log-rank test was used to determine differences in survival between groups analyzed. To compare response rates between groups, Fisher's exact test (two-tailed, 90% confidence interval) was done. A two-tailed paired nonparametric t test (Wilcoxon) was used to compare FLT-PET/CT-derived standardized uptake values before and after GS-9219 treatment. For all statistical analyses, $P < 0.05$ was considered significant.

Results

Subjects. Thirty-eight client-owned (pet) dogs with histologically confirmed spontaneous non-Hodgkin's lymphoma were treated with GS-9219 monotherapy. Patient demographics, tumor stage (22), immunophenotype, and prior chemotherapy history are presented in Table 2. The majority of dogs ($n = 22$; 58%) had been treated previously for their non-Hodgkin's

lymphoma and had either not responded to their initial protocol or had relapsed following treatment; 6 of these dogs had failed two or more prior treatment protocols. Seventeen dogs were treatment naive.

Pharmacokinetics. Pharmacokinetic data are summarized in Table 3. Pharmacokinetic sampling for plasma and PBMC exposure of GS-9219 and its metabolites was done in a subset of animals. In 8 animals treated with a 0.82 mg/kg dose on once every 14 days or once every 21 days regimens (cohort 3-high and cohort 4-high, respectively), the plasma exposures ($AUC_{0-\infty}$) for GS-9219 and cPrPMEDAP were 2,910 and 3,700 nmol/L h, respectively. GS-9219 was rapidly eliminated from plasma with a half-life of <0.5 h, whereas cPrPMEDAP persisted with a half-life of ~4 h. The cytotoxic metabolite PMEG was not observed in plasma samples from any dog.

In PBMCs taken 24 h after dosing during once every 14 days or once every 21 days regimens and 24 h after day 5 dosing in the daily × 5 regimen, total levels (following dephosphorylation) of cPrPMEDAP and PMEG were measured. In contrast to plasma, high concentrations of PMEG were readily detectable in PBMCs. Levels in the 0.82 mg/kg once every 14 days or once every 21 days groups were 456 and 4,020 nmol/L for cPrPMEDAP and PMEG, respectively. Following the final dose on day 5 in dogs dosed with 0.20 mg/kg for 5 days (cohort 1-low), levels were comparable with those observed in the 0.82 mg/kg single-dose groups (mean PBMC levels in 6 dogs of 232 and 2,120 nmol/L for cPrPMEDAP and PMEG, respectively).

Safety evaluation. Adverse events during treatment cycles (summarized in Table 4), when observed, were manageable, reversible, and dose and regimen dependent. Grade 3 adverse events were similarly transient and reversible and were limited to transient hyperbilirubinemia (5 dogs), dermatopathy (4 dogs), anorexia (3 dogs), neutropenia (2 dogs), and diarrhea, increased ALP, dehydration, or fever (1 dog each). Grade 4 adverse events were limited to lethargy, nausea, and neutropenia (1 dog each), all of which were reversible.

DLTs were dermatologic, gastrointestinal, and neutropenia (Table 4). Adverse events were more common in the regimens with the highest dose intensities (0.29 mg/kg/d × 5 and 0.82 mg/kg/wk). Dermatologic toxicity occurred in 14 (37%) dogs; 6 dogs had grade 1, 4 dogs had grade 2, and 4 dogs had grade 3 severity. This toxicity occurred predominantly in the daily × 5 and weekly cohorts, whereas only 2 cases occurred in the once every 14 days cohort and none occurred in the once every 21 days cohort (Table 4). Grade 1 and 2 events consisted of local

superficial erythematous lesions that were often pruritic and most often localized to the ear canal and/or as a small foci on the back or inguinal areas. Grade 3 lesions occurred in 4 dogs, 11% of treated dogs, 2 of which required a dose delay, 1 in the high-dose 0.29 mg/kg/d × 5, and 1 in the 0.82 mg/kg/wk dose cohort. In these latter 2 dogs with grade 3 dermal toxicity, the lesions were more extensive, became exudative, and had bullae formation. All dogs with grade 2 or 3 dermatologic toxicity were treated symptomatically with oral antibiotics, analgesics, and pentoxifylline. The lesions resolved clinically in all cases within 2 to 3 weeks following interruption of GS-9219

treatment and did not recur following retreatment at more prolonged treatment intervals.

Two dogs developed signs of pulmonary dysfunction after they had been withdrawn from study after completing all GS-9219 treatments and had subsequently received other chemotherapy. These pulmonary changes had an unclear relationship to treatment with GS-9219, as both affected dogs were exposed to other cytotoxic chemotherapeutics after withdrawal from this study as well as in 1 dog before enrollment. Both dogs had clinical (tachypnea and dyspnea) and radiographic evidence of pulmonary disease, and 1 dog evaluated by necropsy had

Table 4. Incidence of common adverse events in dogs with naturally occurring non-Hodgkin's lymphoma treated with GS-9219 monotherapy with different dosing schedules

Adverse event and grade	Dose and regimen							
	Daily × 5 d every 21 d		Once every 7 d		Once every 14 d		Once every 21 d	
	0.20 mg/kg (n = 7)	0.29 mg/kg (n = 6)	0.66 mg/kg (n = 3)	0.82 mg/kg (n = 6)	0.66 mg/kg (n = 3)	0.82 mg/kg (n = 6)	0.66 mg/kg (n = 3)	0.82 mg/kg (n = 4)
Anorexia								
1	3	2	1	4	2	2		2
2	1	1						
3	1	1		1				
Emesis								
1	3	2				1		
2	1		1					
Nausea								
1	2					2		
4	1							
Diarrhea								
1	2	3	1	3		3		1
2	1	1	1					
3				1				
Weight loss								
1	1	1		3	1			1
2	2	2						
Lethargy								
1	6			4		3		1
2		1			1			1
4				1				
Neutropenia								
1	1	1				1		
2		2					1	
3		1		1				
4		1						
Dermatopathy*								
1	1	2	1	2				
2	1	2		1				
3		1		1	1	1		
Hyperbilirubinemia [†]								
1		1		1		2	1	
2	1	1		1		2		
3				1	1			3
Glucosuria								
1	1			1	1	1		
2						1		
Proteinuria								
1	1				1	2		2
2								1

NOTE: Incidence is number of dogs with adverse event; if event was observed more than once, only the greatest severity event is listed.

*Dermatopathy consisted most commonly of focal otitis externa or focal erythemic skin lesions in the ventrum. Four dogs developed grade 3 skin lesions that resulted in a delay in treatment in two of the dogs; these lesions resolved with symptomatic treatment in each animal.

[†]Hyperbilirubinemia noted was transient and spurious in nature, as a correlation to the timing of GS-9219 administration was not consistent, and in nearly all instances, values were normal at time points both just preceding and following the noted change.

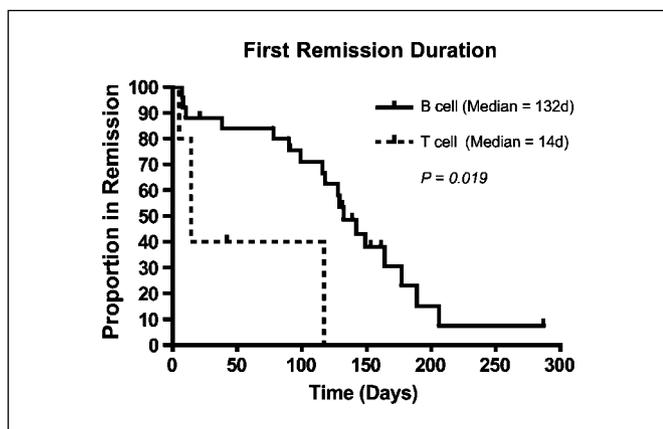


Fig. 1. Kaplan-Meier overall median FRD in 38 dogs with B-cell or T-cell non-Hodgkin's lymphoma receiving GS-9219 monotherapy.

confirmed pulmonary fibrosis. A third dog had hyperplasia of pulmonary epithelium noted on routine follow-up postmortem examination but had no clinical evidence of pulmonary dysfunction. No other dogs in this study had evidence of pulmonary dysfunction as assessed clinically in all dogs, by radiographic analysis in 11 dogs and by postmortem examination in 18 other dogs.

Clinical outcome and efficacy. A minimum of 12 months follow-up has occurred for all dogs. All but 3 dogs experienced relapse or progression of their disease; 2 are still alive in CR (504 and 391 days from treatment initiation) and 1 died of an unrelated cause while in CR 42 days following GS-9219 treatment. One of the long-term survivors had been previously treated with chemotherapy and one was treatment naive before receiving GS-9219. In all, 31 dogs have died and 7 are currently alive. Thirty (79%) dogs receiving GS-9219 monotherapy achieved clinical remission [23 (61%) CR and 7 (18%) PR] and responses were noted in all eight treatment cohorts (Table 1). All 17 naive dogs (previously untreated) experienced a tumor response (13 CR and 4 PR; 100%) to GS-9219 versus 13 of 21 (10 CR and 3 PR; 62%) dogs having previous treatment before GS-9219 ($P = 0.005$). Twenty-five of 29 (86%) tumors with B-cell immunophenotype responded versus 5 of 9 (56%) tumors of T-cell immunophenotype ($P = 0.071$).

The Kaplan-Meier estimate of median FRD for all dogs was 128 days. The median FRDs for dogs with naive versus previously treated non-Hodgkin's lymphoma were 142 and 99 days, respectively ($P = 0.159$). Median FRDs for dogs with B-cell versus T-cell immunophenotypic tumors were 132 and 14 days, respectively (Fig. 1; $P = 0.019$).

FLT-PET/CT. Mean FLT uptake was high in lymphoid tissues in all 9 dogs scanned before GS-9219 treatment. Of the 9 dogs scanned, 7 experienced clinical responses to GS-9219 and underwent post-treatment scans. In these 7 dogs, pretreatment FLT uptake was significantly higher before treatment than after treatment [mean maximum body mass standardized uptake values of 9.8 (2.6-22.3) and 3.5 (1.1-7.9), respectively; $P = 0.016$] as well as 1 month after completion of all five cycles of GS-9219 [mean maximum body mass standardized uptake value of 2.4 (1.5-3.4); $P < 0.031$; Fig. 2].

The presumed clinical response was confirmed by significant reduction in FLT uptake and return to normal nodal image size in all 7 dogs in at least one follow-up FLT-PET/CT scan (see Fig. 2).

Retreatment with GS-9219 monotherapy. In 10 dogs that achieved complete remission and completed five initial cycles of therapy but then experienced relapse of non-Hodgkin's lymphoma, owners chose to attempt induction of a second remission with GS-9219 monotherapy (0.82 mg/kg once every 21 days for five planned cycles). This retreatment protocol appeared to be the best tolerated while providing reasonable efficacy based on cases already entered into the trial. Seven (70%) dogs achieved a second GS-9219 induced remission (6 CR and 1 PR), with a median second remission duration of 65 days. No new adverse events were detected following retreatment.

Discussion

The demographics, tumor stage, and immunophenotypic breakdown of subjects in this study population were similar to previous reports of pet dogs with multicentric non-Hodgkin's lymphoma (16). The majority of dogs in the study were heavily pretreated with chemotherapy protocols commonly in use for non-Hodgkin's lymphoma and, as such, likely represent similar drug-resistant populations enrolled in phase I trials of novel agents in humans.

Despite its short half-life in plasma, GS-9219 was effective at loading lymphoid cells with high levels of the cytotoxic metabolite, PMEG diphosphate. Furthermore, GS-9219 administration resulted in selective PMEG exposure to lymphoid cells. Based on the poor selectivity observed following the direct administration of PMEG in prior studies (3-5), selective intracellular exposure to PMEG and its active diphosphorylated metabolite is likely critical to the therapeutic window observed in this study. The previously observed (6) long intracellular half-life of PMEG diphosphate was evident in the observation that a single high-dose or five daily low doses of GS-9219 resulted in similar intracellular levels. The observations of clinical responses in a majority of dogs in different dosing regimens suggests that the low micromolar concentrations of total PMEG observed in PBMCs in this study were sufficient to cause substantial anticancer activity in lymphoid malignancies.

The antitumor activity observed in canine non-Hodgkin's lymphoma with GS-9219 warrants further study in humans. The CR rates reported here were based on the criteria commonly used in veterinary patients (normalization of peripheral lymphadenopathy with respect to size and cytologic assessment) rather than more stringent criteria employed in human oncology that include CT and/or PET imaging (19, 20). Although the assessment of response based on the veterinary "standard of care" likely overstates response rates in comparison with the "standard of care" used in human clinical trials, confirmation of remission in all 7 of 23 dogs achieving clinical CR that underwent FLT-PET/CT supports the likelihood of the significant responses reported. The results of FLT-PET/CT have utility in monitoring response to treatment and prediction of outcome in humans with non-Hodgkin's lymphoma (23, 24). The profound and rapid decrease in FLT uptake observed 5 days following GS-9219 in dogs with non-Hodgkin's lymphoma in this study is also consistent with the previously observed lymphoid depletion and antiproliferative activity (as measured by Ki-67 index) in lymphoid tissues from normal dogs treated with GS-9219 (6).

The activity of GS-9219 observed in this study compares favorably with other single-agent chemotherapy agents

employed for treating non-Hodgkin's lymphoma in pet dogs (16, 25). In particular, GS-9219 is clearly superior to the only other nucleotide analogues for which single-agent response data are available in pet dogs with non-Hodgkin's lymphoma; gemcitabine and cytosine arabinoside have published single-agent response rates of <7% (26, 27). Additionally, the median FRD for dogs with relapsed non-Hodgkin's lymphoma observed following GS-9219 monotherapy is nearly double that reported previously in dogs with relapsed non-Hodgkin's lymphoma treated with other single-agent or standard multiagent chemotherapy protocols (16, 28–33).

The lower response rate and shorter median FRD observed in dogs with T-cell non-Hodgkin's lymphoma following GS-9219 treatment was not unexpected as the T-cell immunophenotype is a well-documented negative predictor for response to currently available chemotherapeutics in dogs (16). However, based on this experience, the initial human clinical trials of GS-9219 will be conducted in patients with B-cell malignancies.

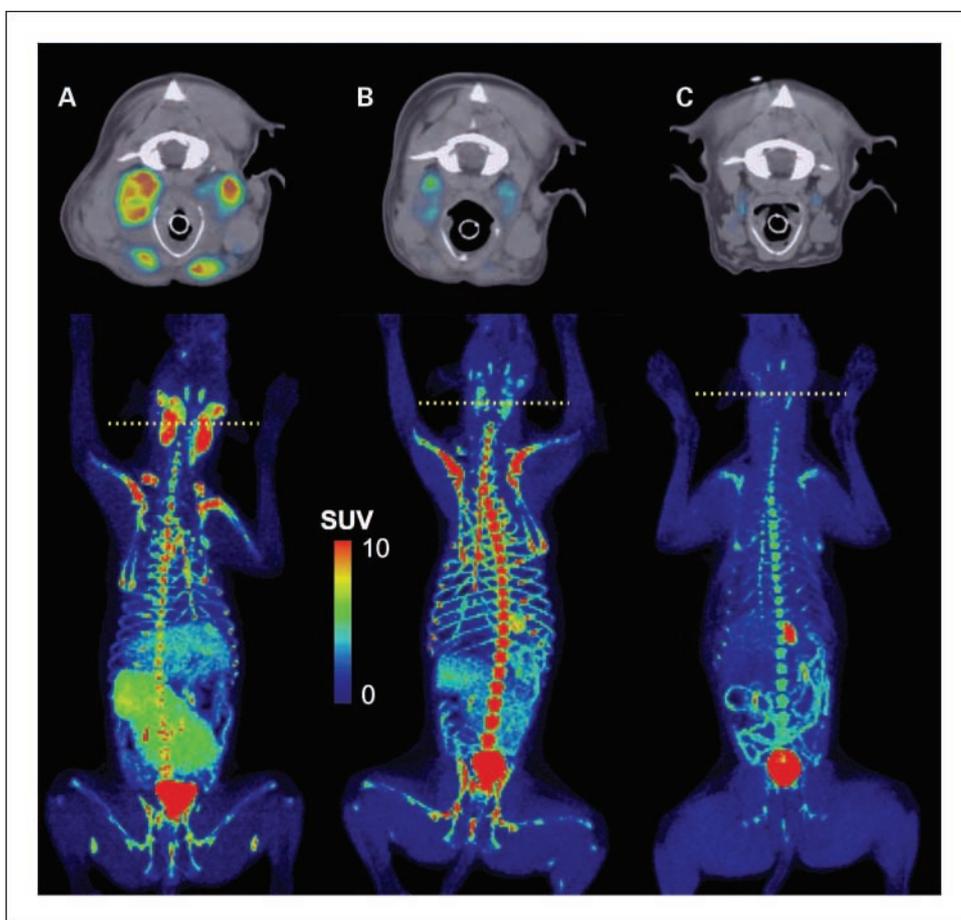
Adverse events were both dose and schedule dependent and all were manageable and reversible. All low-dose protocols were generally well tolerated whether given daily for 5 consecutive days every 21 days or as a single dose given once every 7, 14, or 21 days. The high-dose 5 consecutive day protocol was found to be poorly tolerated due to dose-limiting dermatologic events that developed after the second 21-day cycle. The highest dose used (0.82 mg/kg) was well tolerated at 14- and 21-day treatment intervals but was not tolerated at weekly intervals due to both gastrointestinal and dermatologic dose-limiting

adverse events. Based on this experience, we anticipate that mucocutaneous and hematologic toxicities will be dose-limiting in man. Gastrointestinal toxicities are also likely to occur but can probably be managed with standard supportive measures. The relationship between GS-9219 administration and pulmonary changes that occurred in 3 dogs is unclear and warrants close observation of pulmonary function in subsequent human investigations.

Major antitumor responses were noted in all dose and schedule cohorts employed. Because significant antitumor activity was seen with once every 21-day dosing, the initial human clinical trial will use this regimen. This regimen using intermittent bolus administration will also permit better characterization of GS-9219 toxicities in man. However, given the long intracellular half-life of PMEG following GS-9219, evaluation of regimens using more frequent administration to produce more prolonged drug exposure are potentially attractive. The current study was limited by a lack of randomization into treatment groups and insufficient numbers in each cohort to compare efficacy of the varied schedules used; a larger randomized study with more stringent eligibility and response criteria is currently ongoing in dogs with non-Hodgkin's lymphoma who have failed a single doxorubicin-containing regimen to evaluate the effect of schedule on treatment and toxicity. The results of this study could prompt evaluation of other treatment regimens.

In summary, we describe a preclinical proof-of-concept investigational trial of GS-9219, a novel double prodrug of PMEG, in a relevant large animal model of spontaneous

Fig. 2. Representative FLT-PET/CT of a dog with non-Hodgkin's lymphoma before and after GS-9219 treatment. FLT-PET/CT scan before (A) and 5 d after a single 0.66 mg/kg dose of GS-9219 (B) in a dog with stage V, B-cell non-Hodgkin's lymphoma. A third scan was done 3 wk following completion of five cycles of GS-9219 (C). *Bottom*, whole-body FLT-PET/CT scans at three time points; *top*, cross-sectional FLT-PET/CT. Whole-body PET scan before therapy (A) shows significant proliferative response in affected lymphoid tissues (popliteal, mesenteric, mediastinal, prescapular, submandibular lymph nodes, and spleen). Note that the signal in the urinary bladder and renal calyces is normal and represents urinary excretion of the tracer. Low-level signal is present in the vertebral bone marrow and the gastrointestinal tract and reflects background uptake of tracer in the proliferating cell populations in these tissues. Scans repeated 5 d after initial therapy (B) and 3 wk following completion of all treatment cycles (C) clearly indicate biological response as measured by significantly diminished uptake of tracer; note that biological precedes complete anatomic response when comparing the 5-d post-treatment scan (B) with that at completion of chemotherapy (C).



naturally occurring non-Hodgkin's lymphoma. This study has provided potentially valuable information before beginning human investigations: adverse events were generally transient, manageable, and dose and schedule dependent; the possibility of treatment-related pulmonary toxicity resulted in increased clinical monitoring in the initial phase I trial in man; the active form of the drug persisted selectively in PBMC, prompting further evaluation regimens using more frequent administration; and a favorable therapeutic index was shown in both treatment-naïve and refractory cases of non-Hodgkin's lymphoma in pet dogs. GS-9219 is a promising development candidate and phase I investigations of GS-9219 in human patients with lymphoid malignancies are in progress.

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Disclosure of Potential Conflicts of Interest

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