Clinical Trials: Targeted Therapy

Clinical Cancer Research

NCI Comparative Oncology Program Testing of Non-Camptothecin Indenoisoquinoline Topoisomerase I Inhibitors in Naturally Occurring Canine Lymphoma

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Abstract

Purpose: Only one chemical class of topoisomerase I (TOP1) inhibitors is FDA approved, the camptothecins with irinotecan and topotecan widely used. Because of their limitations (chemical instability, drug efflux-mediated resistance, and diarrhea), novel TOP1 inhibitors are warranted. Indenoisoquinoline non-camptothecin topoisomerase I (TOP1) inhibitors overcome chemical instability and drug resistance that limit camptothecin use. Three indenoisoquinolines, LMP400 (indotecan), LMP776 (indimitecan), and LMP744, were examined in a phase I study for lymphoma-bearing dogs to evaluate differential efficacy, pharmacodynamics, toxicology, and pharmacokinetics.

Experimental Design: Eighty-four client-owned dogs with lymphomas were enrolled in dose-escalation cohorts for each indenoisoquinoline, with an expansion phase for LMP744. Efficacy, tolerability, pharmacokinetics, and target engagement were determined.

Results: The MTDs were 17.5 mg/m^2 for LMP 776 and 100 mg/m^2 for LMP744; bone marrow toxicity was dose-limiting; up to 65 mg/m^2 LMP400 was well-tolerated and MTD was not reached. None of the drugs induced notable diarrhea. Sustained tumor accumulation was observed for LMP744; γH2AX induction was demonstrated in tumors 2 and 6 hours after treatment; a decrease in TOP1 protein was observed in most lymphoma samples across all compounds and dose levels, which is consistent with the fact that tumor response was also observed at low doses LMP744. Objective responses were documented for all indenoisoquinolines; efficacy (13/19 dogs) was greatest for LMP744.

Conclusions: These results demonstrate proof-of-mechanism for indenoisoquinoline TOP1 inhibitors supporting their further clinical development. They also highlight the value of the NCI Comparative Oncology Program (https://ccr.cancer.gov/Comparative-Oncology-Program) for evaluating novel therapies in immunocompetent pets with cancers. *Clin Cancer Res; 24(23); 5830–40. ©2018 AACR.*

Introduction

DNA topoisomerase I (TOP1) is an essential enzyme that relaxes DNA supercoiling by generating single-strand breaks in the DNA backbone, forming reversible cleavage complexes (TOP1cc) under physiologic conditions (1). Camptothecin analogues, such as topotecan and irinotecan, damage DNA by

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Translational Relevance

Only one chemical class of topoisomerase I (TOP1) inhibitors is FDA approved, the camptothecins with irinotecan and topotecan widely used. Because of their limitations (chemical instability, short half-life, drug efflux-mediated resistance, and diarrhea), novel TOP1 inhibitors are warranted. Indenoisoquinoline non-camptothecin topoisomerase I (TOP1) inhibitors were developed as nanomolar inhibitors of TOP1, which overcome chemical instability and drug resistance that limit camptothecin use. Three indenoisoquinolines, LMP400 (indotecan), LMP776 (indimitecan), and LMP744, were examined in a phase I study for lymphoma-bearing dogs to evaluate differential efficacy, pharmacodynamics, toxicology and pharmacokinetics. Objective responses were documented for all indenoisoquinolines; efficacy (13/19 dogs) was greatest for LMP744. Moreover, LMP744 showed remarkable tumor accumulation and retention. These results demonstrate proof-of-mechanism for indenoisoquinoline TOP1 inhibitors supporting their further clinical development. They also highlight the value of the NCI Comparative Oncology Program (https://ccr.cancer.gov/Comparative-Oncology-Program) for evaluating novel therapies in immunocompetent pets with cancers.

trapping the TOP1cc (2, 3), and have demonstrated significant antitumor activity. Topotecan is FDA-approved as therapy for ovarian, small-cell lung, and cervical cancer while irinotecan is approved to treat colorectal and gastric cancers. However, the efficacy of camptothecin derivatives is limited by chemical instability of the camptothecin alpha-hydroxy-lactone E-ring (Fig. 1A), resulting in tight binding to albumin thereby limiting the availability of active drug, and by the rapid reversibility of the camptothecin-trapped TOP1 cleavage complexes (4–6). In addition, efficacy of camptothecins is hindered by development of drug resistance through active drug efflux from cells by ABCG2 efflux pumps (7), and by the short half-life of camptothecins (1–2 hours). Moreover, irinotecan can produce severe diarrhea. To overcome these limitations, indenoisoquinolines were discovered by mining the NCI60 drug database (8, 9). They are in clinical development as a class of noncamptothecin TOP1 inhibitors (10).

To date, more than 500 indenoisoquinoline candidates have been synthesized and assessed for selective activity against TOP1. Three lead compounds, LMP400 (NSC724988; indotecan), LMP776 (NSC725776; indimitecan; refs. 11, 12) and LMP744 (MJ-III-65; ref. 13; Fig. 1B), have been selected for development (10). In addition to the chemical stability of the indenoisoquinolines due to their lack of E-ring hydroxylactone, the selection was based on the following criteria: (i) potency against recombinant TOP1, (ii) ability to induce cleavage at different sites from camptothecins, (iii) resulting TOP1cc that are more stable than those of camptothecins, (iv) potent antiproliferative activity shown in the NCI 60 cell-line screen, (v) selectivity for TOP1 in cleavage complexes, (vi) activity dependent on TOP1 ($TOP1^{-/-}$ cells are resistant), and (vi) ability to induce γH2AX foci, an early marker of DNA double strand breaks, at pharmacologic concentrations (10–12, 14–16). *In vitro* and *in vivo* studies have demonstrated that these indenoisoquinolines exert their antineoplastic effects through TOP1 inhibition and have elucidated the cleavage sites and stability of the cleavage complexes (9, 13, 17, 18). An immunofluorescence assay for γH2AX has been developed as a biomarker of chemotherapy-induced DNA damage (11, 12), and has been used to demonstrate that γH2AX foci occur *in vivo* subsequent to treatment with indenoisoquinolines (17, 19, 20).

The US National Cancer Institute's Center for Cancer Research launched the Comparative Oncology Program (COP) to assess novel treatments for humans and provide pet owners access to cutting-edge treatment for their dogs and cats (https://ccr.cancer.gov/Comparative-Oncology-Program/pet-owners/about). Comparative oncology studies, defined as the conduct of preclinical research in tumor-bearing companion animals, offer a novel opportunity to obtain critical data regarding pharmacokinetic/pharmacodynamic properties and biomarker validation in the drug development process that may not be achieved in traditional preclinical models or in early-phase studies in human subjects. In veterinary medicine, brief sedation or general anesthesia are commonly employed to facilitate diagnostic testing, such as imaging, tissue sampling, and bone marrow aspirates. Also, additional blood sampling can be integrated in comparative oncology protocols to potentially allow for identification of circulating surrogate biomarkers and eliminate the need for repeated tissue biopsies. Therefore, incorporation of serial blood sampling and tumor biopsies to validate potential biomarkers of drug exposure in comparative oncology research protocols is feasible, tolerable by the patient, and generally acceptable to the owners, and can provide the ability to better assess tumor and normal tissue pharmacodynamic modulation to define a pharmacodynamic–dose relationships for novel anticancer agents either prior to or in parallel with early-phase human trials (21, 22).

While phase I clinical trials evaluating LMP400 and LMP776 were underway for adults with relapsed solid tumors and lymphoma at the National Cancer Institute, we tested these two indenoisoquinoline derivatives in parallel with a third indenoisoquinoline, LMP744, which also showed promising activity in preclinical models (9, 13, 17). The primary objectives of this early-phase veterinary clinical trial (procedures and schedules are outlined in Table 1) were to determine: (i) an MTD for LMP744 in parallel with LMP400 and LMP776, (ii) the acute toxicity profiles of the three analogues, (iii) their activity against naturally occurring lymphomas in dogs, (iv) the pharmacokinetic properties of the drugs, (v) target engagement by measuring induction of γH2AX foci and modulation of TOP1, and (vi) the pharmacodynamic–dose response relationships for each compound in a naturally occurring, large animal tumor model. A secondary objective was to validate the canine comparative oncology model to inform development of new anticancer therapies.

Materials and Methods

Comparative oncology trials consortium

The Comparative Oncology Trials Consortium (COTC) infrastructure, data reporting, and goals have been previously described. Our canine clinical trial was conducted through a multi-institutional consortium (23, 24). Nine COTC sites, Colorado State University, The Ohio State University, Purdue University, University of California - Davis, University of Missouri, University of Pennsylvania, University of Tennessee, University

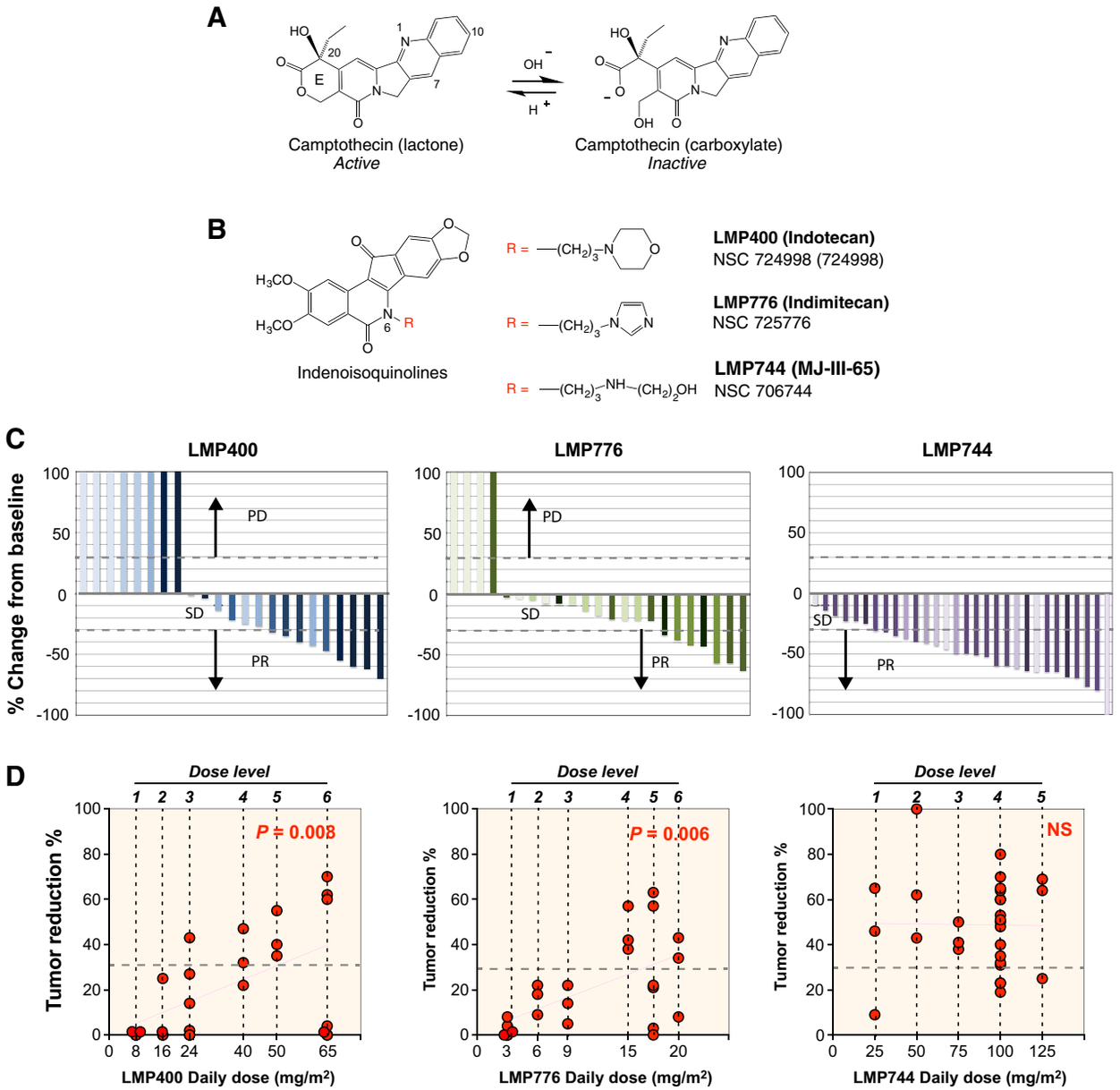


Figure 1.

A and **B,** Chemical structures of the three indenoisoquinolines included in the present study. The structure of camptothecin is shown for comparison, and for demonstrating that the indenoisoquinolines do not bear the alpha-hydroxylactone E-ring of camptothecins. Topotecan and irinotecan are camptothecin derivatives with substitutions at positions 7 and 10 of camptothecin. **C** and **D,** Antitumor activity of the three indenoisoquinolines in dog lymphoma. **C,** Best response plots with darker colors indicating higher doses (see Supplementary Table S2). **D,** Tumor response as a function of dose. Individual dogs are shown as data points (see Supplementary Tables S4–S6). PR is partial response; SD is stable disease; PD is progressive disease. Statistical significance (*P* values) given for relationship of drug dose level to daily dose level.

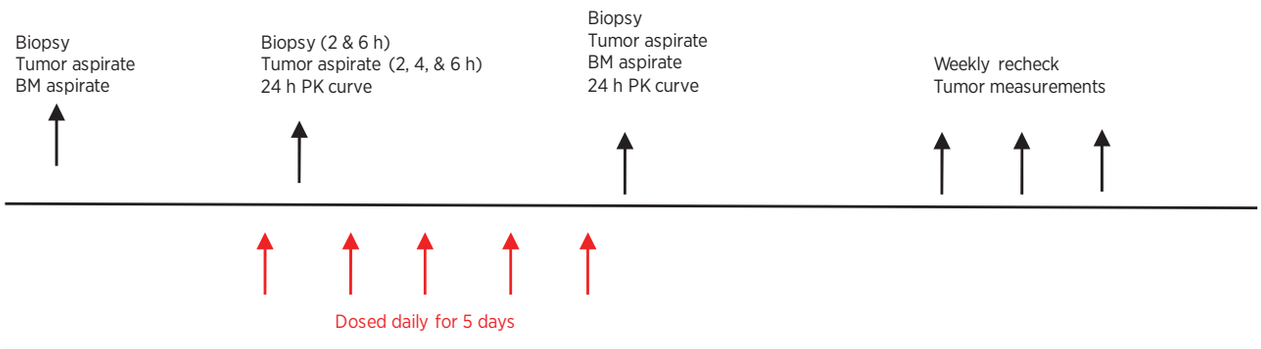
of Tufts, and the University of Wisconsin-Madison, participated in the study, and all dogs recruited were evaluated following a defined protocol and standard operating procedures. The study protocol was reviewed and approved by each participating site's Institutional Animal Care and Use Committee and, where applicable, Clinical Trials Review Board. All study data was managed by the Comparative Oncology Program utilizing the Cancer Central Clinical Database (C3D).

Trial eligibility and enrollment

Client-owned pet dogs weighing ≥15 kg with cytologically or histologically confirmed lymphoma were included in the study. Eligibility criteria required dogs to have a nodal presentation (stage 2 or greater) with at least three nodes a minimum of 3 cm in longest diameter that were amenable to repeated biopsies. Both newly diagnosed and previously treated dogs were eligible with a 2-week washout for chemotherapy and radiotherapy. Dogs

Table 1. Schedule of patient evaluations and study procedures

Action	Pretreatment	Day 1	Day 2	Days 3 & 4	Day 5	Day 6	Days 8, 15, 22	Day 29
Patient eligibility	X							
Tumor measurements (calipers)	X						X	X
Physical examination	X	X	X	X	X	X	X	X
Digital photo of tumor	X						X	X
CBC/chemistry/UA/coagulation profile	X	X			CBC only		X	X
Thoracic radiographs	X							?
Plasma collection		X	X	X	X	X	X	X
Serum collection		X	X		X		X	X
Indenoisoquinoline administration		X	X	X	X			
Tumor biopsy	X	X				X		
Tumor aspirate	X	X				X		
Bone marrow aspirate	X					X		
Owner assessment form	X						X	X



previously treated with corticosteroids or L-asparaginase had a 7-day washout prior to study initiation. All dogs received a physical exam, laboratory evaluations (complete blood count, serum biochemical profile, and urinalysis), and thoracic radiographs as part of the eligibility screening within 10 days prior to study enrollment. Dogs determined to have any significant comorbid illness were excluded. In addition, dogs with any of the following were considered ineligible for enrollment: creatinine >3.0 mg/dL, total bilirubin >2.0 mg/dL or elevated bile acids, HCT <25%, platelets <50,000/μL, or any other >grade 2 hematologic or biochemical abnormality based on the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events (VCOG-CTCAE) v1.1. (25). Eligibility criteria included a performance status of 0 or 1 [modified Eastern Cooperative Oncology Group (ECOG)] and informed owner consent.

Study schema

A schedule of patient evaluations, diagnostics, and treatments performed at each study time point are summarized in Table 1. The trial design utilized a schedule of five daily infusions of an indenoisoquinoline during a 28-day treatment course. At time of enrollment, dogs were assigned an agent based on sequential order of accrual. All biological collections were identical among the three drug cohorts.

Caliper measurements of the longest dimension of one to five target and up to five non-target lymph node measurements were performed independently by two clinicians and recorded in millimeters at each weekly visit. Lymph nodes had to be a minimum of 20 mm in the longest diameter at baseline to be considered a target lesion. Although not a primary study endpoint, tumor response was assessed at each weekly study visit, and responses determined using the Response Evaluation Criteria for Peripheral Nodal Lymphoma in Dogs v1.0. (26) Progressive disease (PD) was defined as at least a 20% increase in the sum of the mean lymph node measurements or the development of new lesions. Partial response (PR) was defined as a minimal decrease of 30% in the sum of mean lymph node measurements; dogs were considered to have stable disease (SD) if they did not meet the criteria for either PD or PR. Dogs were determined to have a complete response (CR) if all peripheral lymph nodes were determined to be nonpathologic in size by the supervision clinicians. Dogs with an objective response (CR or PR) at day 29 were eligible for subsequent cycles of treatment. Dogs that developed PD at any point or had SD at day 29 were removed from study and allowed to pursue alternative treatments.

Treatment administration and toxicity assessment

The Developmental Therapeutics Program Repository of the NCI provided the three indenoisoquinolines for this study. Dogs received five daily doses of LMP776, LMP400, or LMP744. Drug was filtered, diluted in 5% dextrose in water (D5W), and administered intravenously through a central line over 1 hour. Dogs receiving LMP776 and LMP400 were administered a dose of diphenhydramine 1 mg/kg i.m. 20 minutes prior to the infusion. Dogs assigned to receive LMP744 received an increase in premedication with additional diphenhydramine 2 to 3 mg/kg orally approximately 12 hours, and diphenhydramine 2 mg/kg i.m. prior to each dose. Dogs were hospitalized during the 5-day course of treatment. Dogs had respiratory rate, heart rate, EKG, and blood pressure monitored every 15 minutes during the 60-minute infusion and 30 minutes after completion of the infusion. This level of monitoring was no longer deemed necessary after cohort 2. Hematologic and biochemical evaluations [CBC, chemistry profile, coagulation profile (PT/PTT), urinalysis (UA)] were performed on days 1, 5 (CBC only), 8, 15, 22, and 29 to evaluate toxicities associated with administration of the

three indenoisoquinolines. These diagnostic tests were performed at a central laboratory (Antech GLP, Morrisville), with the exception of the CBC on day 5, which was performed at the COTC site prior to the last drug administration to ensure that neutrophil counts were adequate for treatment.

The doses of the three indenoisoquinolines (LMP776, LMP400, LMP744) were escalated independently in a standard 3+3 dose escalation design. The starting cohort dose was determined to be 15% of the MTD of a 5-day dosing schedule of healthy beagle dogs. Three dogs were enrolled in the first dose cohort and observed for dose-limiting toxicities (DLTs). Severity of toxicity of each indenoisoquinoline was assessed using the VCOG-CTCAE v1.1 (25) at each study time point, and toxicity attributions were designated as due to drug, disease, or other cause. The certainty of attributions was further characterized as unrelated, unlikely, possible, probable, or definite. DLT was defined as any grade 3 nonhematologic or grade 4 hematologic toxicity (25). If no DLTs were observed in the first cohort of three dogs within 2 weeks of indenoisoquinoline administration, a second cohort of dogs was treated at an increased dose. If a DLT was observed in one dog, the cohort was expanded up to a total of six dogs. If no additional DLTs were noted in the expanded cohort of six dogs, dose escalation was continued with a higher dosage of the indenoisoquinoline. If $\geq$2 DLTs were observed in the initial or expanded cohort, case accrual was stopped and the MTD was determined to be the highest dosage used in a cohort where <2 DLTs were noted. Dogs that were removed from the study prior to completion of the five daily infusions were replaced in the cohort to fully assess acute toxicities associated with the indenoisoquinolines. Dose deescalation was permitted to better define the MTD for each agent. Dose cohorts for all three agents are shown in Supplementary Table S2.

Plasma pharmacokinetics

Pharmacokinetic samples were collected over 24 hours (0, 30, 58, 90 minutes, 2, 4, 6, 8, 12, and 24 hours after the start of infusion) on day 1 (first dose) and day 5 (fifth dose) to evaluate dose–exposure relationships of LMP776, LMP400, and LMP744. Additional plasma samples were collected on day 3, 4, 8, 15, 22, and 29. Serum was collected pretreatment on day 2 (24 hours post first drug infusion), day 5, day 6 (24 hours post fifth drug infusion), days 8, 15, 22, and 29. All samples were immediately placed on ice and processed within 20 minutes of collection and stored at −80°C until batch shipment. All pharmacokinetic analyses were performed as described previously (27).

Plasma levels of indenoisoquinolines were determined by LC/MS-MS on a API 3000 spectrophotometer with an Agilent 1100 HPLC. Samples were mixed with acetonitrile containing a deuterated version of the appropriate analyte as an internal standard, centrifuged, and the supernatant transferred, dried, and resuspended in acetonitrile/water/formic acid. LMP400 and LMP776 were eluted from a Synergi Polar RP column and LMP744 was eluted from a Luna phenyl-hexyl column, both with a gradient of acetonitrile/water/formic acid. Calibrators and quality control samples were prepared in blank dog plasma and run with study samples on each day of analysis. Plasma concentration data (0–24 hours) was analyzed by noncompartmental methods using PK Solutions software (Summit Research Services).

Tumor pharmacodynamics and drug levels

Lymph node biopsies were collected from all dogs for histologic characterization and immunophenotyping, for assessment of accumulation of indenoisoquinolines in tumor tissue, and to measure TOP1 and γH2AX levels pre- and posttreatment. Incisional lymph node biopsies were performed in triplicate using a 14G Tru-cut needle prior to treatment and on day 1, 2 hours and 6 hours posttreatment. The largest Tru-cut biopsy was bisected, and these two pieces and the second biopsy sample were flash frozen and stored at −80°C. Twenty-four hours following the fifth drug dose (day 6), dogs had a lymph node extirpated, trisected, and processed as described above. To maximize the quality of these biopsy samples and prevent excessive hemorrhage and necrosis, the biopsies were performed in two separate lymph nodes. The pretreatment and day 6 samples were obtained from the same node (node 1) and the two biopsies performed on day 1 at 2 and 6 hours posttreatment were collected from a second lymph node (node 2). Formalin-fixed samples were shipped on day 6 to Colorado State University for histologic evaluation and immunophenotyping (pretreatment sample only). The frozen samples were held at −80°C until study participation was completed; tissue pharmacokinetic analysis was performed at the University of Pittsburg; tissue TOP1 and γH2AX assessments were performed at the National Cancer Institute's Frederick National Laboratory for Cancer Research using validated microscopy and ELISA, respectively (https://dctd.cancer.gov/researchresources/researchresources-biomarkers.htm). Tumor levels of indenoisoquinolines were determined using a previously described human plasma assay (27) that was cross-validated for canine tumor homogenates. Samples were homogenized on ice in PBS and then extracted with ethyl acetate. Dried residues were resuspended in mobile phase and quantified against calibration curves prepared in human plasma.

Phospho-H2AX (γH2AX) status pre- and posttherapy was also evaluated in lymph node and bone marrow aspirates; these samples were collected from all dogs as outlined in Table 1. Lymph node aspirates were collected using a 22G hypodermic needle and placed into 2-mL Plasmalyte A in a heparinized tube on day −1, day 1 at 2, 4, and 6 hours posttreatment, and on day 6 (24 hours following the fifth dose). Aspirates obtained pretreatment and on day 6 were collected form node 1. Aspirates collected at the 2-hour and 6-hour time points on day 1 were collected from node 2, and the sample obtained at the 4-hour time point was collected from a third lymph node (node 3). Bone marrow aspirates were obtained prior to treatment and day 6. All lymph node and bone marrow aspirates were shipped on the day of collection to the NCI for γH2AX assessment. Tumor samples were obtained as previously described, flash frozen, and stored at −80°C until shipment. Immunofluorescence assays for γH2AX were performed as previously described for all tissues (12).

LMP744 dose expansion cohort. To gain additional pharmacokinetic and efficacy data for LMP744, an expansion of the 100 mg/m^2 cohort (cohort 5) was performed; an additional 13 dogs were treated at this dose level, one of which was subsequently removed from study because baseline lymph nodes biopsies showed no evidence of disease. The plasma and serum sampling schedule was the same as described for the dogs enrolled in the doseescalation portion of the study. To assess variability between basal TOP1 and γH2AX between lymph nodes, pretreatment biopsy samples were collected from both node 1 and node 2. The day 1, 2-hour and 6-hour biopsy samples were collected from node 2 and node 1, respectively. A biopsy was added on day 2 prior to the second dose of LMP744 to evaluate tissue retention of the drug 24 hours after

Table 2. Histologic classification of lymphoma for all dogs

Histology	
Diffuse large B cell	29 (34.5%)
Marginal zone (B cell)	24 (28.6%)
Peripheral T cell	8 (9.5%)
NS[a]	5 (6.0%)
Burkitt-like	4 (4.8%)
Lymphoblastic T cell	4 (4.8%)
Lymphoblastic B cell	2 (2.4%)
Small lymphocytic lymphoma (T cell)	2 (2.4%)
Small lymphocytic lymphoma (B cell)	2 (2.4%)
Small lymphocytic lymphoma (immune unk)	2 (2.4%)
B cell, NOS[b]	1 (1.2%)
Lymphoma, NOS	1 (1.2%)

[a]NS, insufficient sample.
[b]NOS, not otherwise specified.

the first treatment, and to provide a baseline for direct comparison with day 6 drug levels. Bone marrow aspirates were not collected from dogs enrolled in the dose-expansion cohort.

Results

Patient population

Eighty-four dogs were enrolled from May 2012 to July 2015 at the nine participating COTC institutions. Thirteen of these dogs were enrolled in the dose-expansion cohort for LMP744, and the remainder were enrolled in the dose escalation cohorts for the three indenoisoquinolines. The age, sex, weight, and breed for all dogs enrolled in the study are reported in Supplementary Table S1. Fifty dogs (59.5%) had received no lymphoma-specific treatment prior to enrollment into the study. Nineteen dogs (22.6%) had previously received treatment with corticosteroids or L-asparaginase, and 15 dogs (17.9%) had previously been treated with cytotoxic chemotherapy. The histologic classification of the lymphomas is reported in Table 2. The dominant tumor types were diffuse large B-cell and marginal zone (B-cell) lymphoma (34.5 and 28.6%, respectively).

Determination of MTD

Twenty-seven dogs received LMP400 in one of six dose cohorts (Supplementary Table S2). The maximum drug dose evaluated was 65 mg/m^2. One of six dogs treated at this dose developed DLTs consisting of grade 4 neutropenia and thrombocytopenia on day 13. One dog in the 24 mg/m^2 cohort went into cardiopulmonary arrest on day 5 prior to infusion of the agent when sedated for replacement of a central intravenous line; a definitive cause of death was not determined on necropsy but was thought most likely to be due to an arrhythmia based on the Boxer breed of dog. The cohort was expanded to six dogs, and no further DLTs were observed.

Twenty-four dogs received LMP776 in one of six dose cohorts (Supplementary Table S2). The MTD of this agent was 17.5 mg/m^2 given once daily for five consecutive days in a 28-day cycle. A grade 5 adverse event occurred for a dog in the first cohort (3 mg/m^2) immediately following the first infusion of drug. A necropsy was performed, and the acute respiratory decompensation and subsequent death was attributed to the dog's advanced stage of disease and heavy burden of lymphoma in the pulmonary parenchyma. Two dogs in the fifth dose cohort (20 mg/m^2) developed DLTs consisting of grade 4 neutropenia and thrombocytopenia on day 9 of the study. One of these dogs also developed grade 3 anorexia and vomiting. To further refine the MTD, a sixth cohort of dogs was treated at a reduced dose (17.5 mg/m^2), and one out of six dogs in the sixth cohort developed a DLT consisting of grade 3 anorexia and diarrhea.

Twenty dogs received LMP744 in one of five dose cohorts (Supplementary Table S2). The MTD of this agent was determined to be 100 mg/m^2. At a dose of 125 mg/m^2, two dogs developed dose-limiting toxicities that were attributable to the drug. One dog developed grade 4 neutropenia and thrombocytopenia on day 10; a second dog developed a grade 4 neutropenia on day 11. Two dogs in the 125 mg/m^2 cohort were unable to be assessed for response and toxicity because they were removed from study prior to completion of the course of treatment. One dog was removed on day 1 due an aspiration pneumonia secondary to the sedation/anesthesia for the biopsy procedure, and a second dog was removed on day 3 because of grade 3 hypersensitivity during the infusion.

Acute toxicity profiles and dose-limiting bone marrow toxicity

Adverse events for all three agents are summarized in Supplementary Table S3. Gastrointestinal (GI) toxicity, such as anorexia, nausea, diarrhea, and vomiting, occurred commonly for all three indenoisoquinolines and was most often mild to moderate in severity. GI toxicity occurred with greater frequency and severity for LMP744; 5 dogs experienced grade 3 anorexia. Hematologic toxicities were also common for all three agents and were the DLTs for both LMP776 and LMP744. Of the 6 grade 4 neutropenias and 4 grade 4 thrombocytopenias that developed in dogs treated with LMP744, 3 of each of these occurred in the dose-expansion phase.

Hypersensitivity reactions were not observed for LMP776 or LMP400, but grade 1 ($n = 2$), grade 2 ($n = 3$), and grade 3 ($n = 1$) hypersensitivity reactions were observed for LMP744, despite the additional diphenhydramine administered to these patients prior to each drug infusion. Two dogs experiencing hypersensitivity reactions required parenteral administration of dexamethasone to mitigate these events and continue treatment of LMP744. Elevations of liver enzymes including ALP, ALT, and AST also occurred with greater frequency and severity for LMP744 than for the other two agents, though a grade 3 AST elevation was noted in one dog treated with LMP776. One dog treated with LMP744 at 50 mg/m^2 developed a grade 4 ALP increase; this dog also experienced hypersensitivity reactions during drug infusion and had high doses of dexamethasone administered daily to allow for continuing treatment. As such, the grade 4 ALP was attributed to the administration of high-dose corticosteroids during the study period.

Response assessment and efficacy of the three indenoisoquinolines

Objective responses were observed for all three indenoisoquinolines, particularly at the higher dose cohorts for LMP400 and LMP776. Notably, patient responses were documented in all 5 dose cohorts for LMP744 (Fig. 1; Supplementary Table S2). For all dogs in the dose escalation cohorts that experienced SD, PR, or CR ($n = 59$), the day of maximal response to treatment was day 8 for 39 dogs (66.1%), day 15 for 15 dogs (25.4%), day 22 for four dogs (6.8%), and day 29 for one dog (1.7%). Responses were generally short-lived with a median duration of response of 15 days (range, 8–51 days) for all dogs. The three dogs remaining in PR at day 29 of the first cycle received a second cycle of drug; one dog each received a second cycle of LMP776 15 mg/m^2, LMP400 65 mg/m^2,

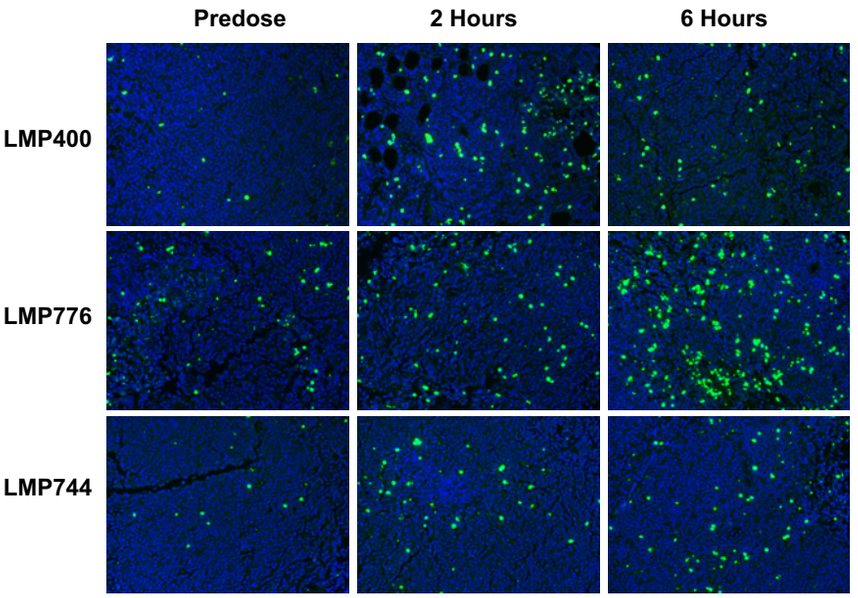


Figure 2.
Representative histone γH2AX response in lymphoma biopsies determined by quantitative immunofluorescence assay for three individual dogs as function of time following treatment with the indenoisoquinolines.

and LMP744 25 mg/m^2. All maintained a PR through day 22 of the second cycle, at which time progressive disease was noted for all three dogs.

For the 19 dogs treated with LMP744 100 mg/m^2 (six in dose escalation and 13 in the dose-expansion cohort), 13 dogs (68.4%) had a PR, five (26.3%) had SD, and one (5.3%) was unable to be assessed for response due to early removal from the study because no evidence of malignancy was demonstrated in pretreatment biopsies. Maximal response occurred on day 8 for six dogs (33.3%), day 15 for eight dogs (44.4%), and day 22 for four dogs (22.2%). Median duration of response was maintained through day 15 (range, 8–22 days) for these 18 dogs.

Together, these results show that all three indenoisoquinolines demonstrated therapeutic activity; LMP744 was markedly more effective than either LMP400 and LMP776. Responses to LMP744 occurred even at the lowest dose level administered and without significant toxicity.

Pharmacodynamic analyses demonstrate target engagement

To determine target engagement in response to indenoisoquinoline treatment, we measured histone γH2AX induction and TOP1 downregulation in lymph node biopsies. These are two well-established biomarkers of the DNA damage response to TOP1 inhibitors (11, 12, 18).

γH2AX induction was observed 2 and 6 hours after the first dose of the three indenoisoquinolines (see Table 1 for schedule). Of the 15 dogs evaluated during treatment with LMP400, 12 showed a marked γH2AX increase, while the remaining three dogs showed 0 to 19% changes (Supplementary Table S4; Fig. 2). Only two dogs (one at dose level 1 and the other at dose level 4) demonstrated no increase, which was associated with limited therapeutic responses (Supplementary Table S4). LMP776 treatment produced a significant increase in tumor γH2AX in nine out of 18 dogs analyzed. Eight showed no significant γH2AX increase, which correlated with minimal tumor reduction (Supplementary Table S5; Fig. 2). On the other hand, LMP744 treatment produced a a marked increase in γH2AX levels in 10 of 12 dogs evaluated, while two showed no increase despite a clinical response to therapy (Supplementary Table S6; Fig. 2). The γH2AX results demonstrate that the indenoisoquinolines produce DNA damage in canine lymphomas, as expected for TOP1 inhibitors (12, 15). Also, the γH2AX expression at 6 hours post-drug administration was associated with apoptotic cell death as measured by γH2AX colocalization with cleaved caspase 3 (28). γH2AX activation was therefore primarily measuring apoptotic cell death (14, 16), and did not clearly predict clinical activity for all three of the compounds evaluated, which might be explained by the other death pathways engaged in tumor response.

Regarding TOP1, consistent with their mechanism of action *in vitro*, most dogs treated with the indenoisoquinolines exhibited TOP1 downregulation, a known response to TOP1 targeting (refs. 18, 29; Supplementary Tables S4–S6).

Pharmacokinetic analyses reveal high tumor retention for LMP744

Table 3 summarizes the plasma pharmacokinetics of the three indenoisoquinolines in each dose cohort (see also Supplementary Table S2). LMP776 showed the shortest half-life ($t_{1/2}$ = 5–7 hours) and LMP744 the longest (mean $t_{1/2}$ = 17 hours); LMP400 had an intermediate half-life (10–14 hours). The volumes of distribution (VD) varied widely across the three indenoisoquinolines and were correlated with the plasma half-life. LMP776, which had the shortest half-life had the smallest VD (mean = 98 L/m^2). LMP744, which had the longest half-life, demonstrated the largest VD (1470 L/m^2; i.e., 15-fold larger than LMP776), and LMP400 was intermediate (VD = 708 L/m^2).

Tumor retention was also determined at day 6, one day after the fifth and last daily drug infusion (see scheme at the bottom of Table 1). Notably, marked retention of LMP744 was measured, with drug concentrations in tumors higher on day 6 than 6 hours after the first infusion (Fig. 3A, right; Supplementary Table S6). LMP744 demonstrated little or no accumulation in plasma over the 5-day treatment period (Fig. 3B), suggesting that the increasing tumor levels are a result of slow efflux rather than being driven by increasing plasma levels. LMP776 produced the lowest level of drug in tumor (Supplementary Table S5); and LMP400 showed

Table 3. Mean noncompartmental pharmacokinetic parameters (0- to 24-hour data)

LMP400

Cohort	Dose (mg/m^2)	Dose (mg/kg)	*n*	C_{max} (ng/mL)	$t_{1/2}$ (h)	AUC_{0-t} (ng-h/mL)	AUC_{0-inf} (ng-h/mL)	Cl (L/h/m^2)	V_D (L m^2)
1	8	0.4	3	98.8	13.9	388	492	16.3	569
2	16	0.8	3	163	10.2	709	818	23.3	2,202
3	24	1.2	6	161	12.9	688	848	30.9	454
4	40	2	3	422	9.6	1,511	1,784	32.3	277
5	50	2.5	3	476	11.4	1,401	1,648	30.9	316
6	65	3.25	6	362	10.7	1,404	1,741	39.7	432
Mean					**11.4**			**28.9**	**708**
SD					**1.7**			**8.08**	**739**
CV%					**14.4**			**27.9**	**104**

LMP776

Cohort	Dose (mg/m^2)	Dose (mg/kg)	*n*	C_{max} (ng/mL)	$t_{1/2}$ (h)	AUC_{0-t} (ng-h/mL)	AUC_{0-inf} (ng-h/mL)	Cl (L/h/m^2)	V_D (L/m^2)
1	3	0.15	5	33	4.9	137	142	26.2	172
2	6	0.3	3	75.7	5.6	562	585	11	91.0
3	9	0.45	3	124	7.3	1,135	1,300	8.79	85.6
4	15	0.75	3	270	5.8	1,971	2,185	8.44	59.3
4.5	17.5	0.875	6	317	4.7	1,844	1,970	15.4	88.1
5	20	1.0	3	438	6.4	1,872	2,004	11.4	93.7
Mean					**5.8**			**13.5**	**98.3**
SD					**0.97**			**6.68**	**38.2**
CV%					**16.7**			**49.4**	**38.9**

LMP744

Cohort	Dose (mg/m^2)	Dose (mg/kg)	*n*	C_{max} (ng/mL)	$t_{1/2}$ (h)	AUC_{0-t} (ng-h/mL)	AUC_{0-inf} (ng-h/mL)	Cl (L/h/m^2)	Vss (L/m^2)
1	25	1.25	3	96	13.7	321	413	60.9	929
2	50	2.5	3	124	14.6	467	633	84.0	1,378
3	75	3.75	3[a]	247	14.6	816	1,090	76.8	1,133
3.5	100	5	6	189	20.6	862	1,040	102	2,288
4	125	6.25	3	501	23.4	1,271	1,880	67.4	1,569
Mean					**17**			**79.0**	**1,470**
SD					**6.5**			**25.3**	**767**
CV%					**38**			**32**	**52**

[a]Likely port draw from one dog in cohort; $n = 3$ for $t_{1/2}$ only; other values $n = 2$.

tumor retention at day 6 at levels similar to those detected 6 hours after the first infusion (Fig. 3A, left; Supplementary Table S4).

Together, these results reveal that the three indenoisoquinolines have distinct plasma and tumoral pharmacokinetics, with LMP744 demonstrating prolonged plasma half-life (17 hours), high volume of distribution (1,470 L/m^2), and sustained tumor accumulation.

Discussion

Utilizing the comparative oncology model of companion dogs with cancer to investigate pharmacokinetic/pharmacodynamic properties of novel molecules provides important advantages for therapeutics development (21, 24). Benefits include larger animal size allowing for repeated blood and tissue collection for pharmacokinetic and pharmacodynamic assessment, diversity of tumor microenvironment, thorough evaluation of toxicity through repeated hematologic and biochemical monitoring and owner-reported adverse events, and drug evaluation in patients with an intact host immune system. The goal of the current study was to identify the MTD, determine the acute toxicity profile, describe the pharmacokinetic parameters, and demonstrate proof-of-mechanism for three indenoisoquinolines, LMP776 (indimitecan), LMP400 (indotecan), and LMP744, in a naturally occurring, large animal tumor model.

MTDs were defined in this study for LMP776 and LMP744 as 17.5 mg/m^2 and 100 mg/m^2, respectively; doses up to 65 mg/m^2 of LMP400 were achieved without reaching MTD. These LMP400 doses are comparable with those defined as maximally tolerated in the phase I human trial of LMP400 (60 mg/m^2 daily for 5 days) in heavily pretreated human patients (11), suggesting that individuals with no prior chemotherapy may tolerate the bone marrow toxicity of the indenoisoquinolines better. Similar to other TOP1 inhibitors, such as topotecan and irinotecan, hematologic toxicities, namely neutropenia and thrombocytopenia, were dose-limiting for LMP776 and LMP744. Gastrointestinal adverse events were also reported for all three agents, and while generally mild to moderate, dogs receiving LMP744 experienced gastrointestinal adverse events more frequently and of greater severity. Development of mild to moderate gastrointestinal adverse events were not unexpected because grade 1 and 2 nausea, vomiting, and diarrhea occur with frequency in humans receiving topotecan on a similar 5-day dosing schedule (30–32). In addition, a pilot study evaluating the pharmacokinetcis and γH2AX modulation by topotecan in pet dogs demonstrated mild to moderate gastrointestinal signs as well.

Delayed diarrhea, which is the most severe nonhematologic dose-limiting toxicity for irinotecan administration (33, 34), was not observed with LMP744 or the two other indenoisoquinolines. Similarly, diarrhea was not reported as a severe toxic event in the human clinical trial of LMP400 (11). Together, our results reveal that bone marrow toxicity, and not significant diarrhea, is dose limiting for the three indenoisoquinoline analogues studied in this dog lymphoma comparative oncology trial.

All three indenoisoquinolines demonstrated therapeutic activity. LMP776 was the least active in the dog lymphoma model.

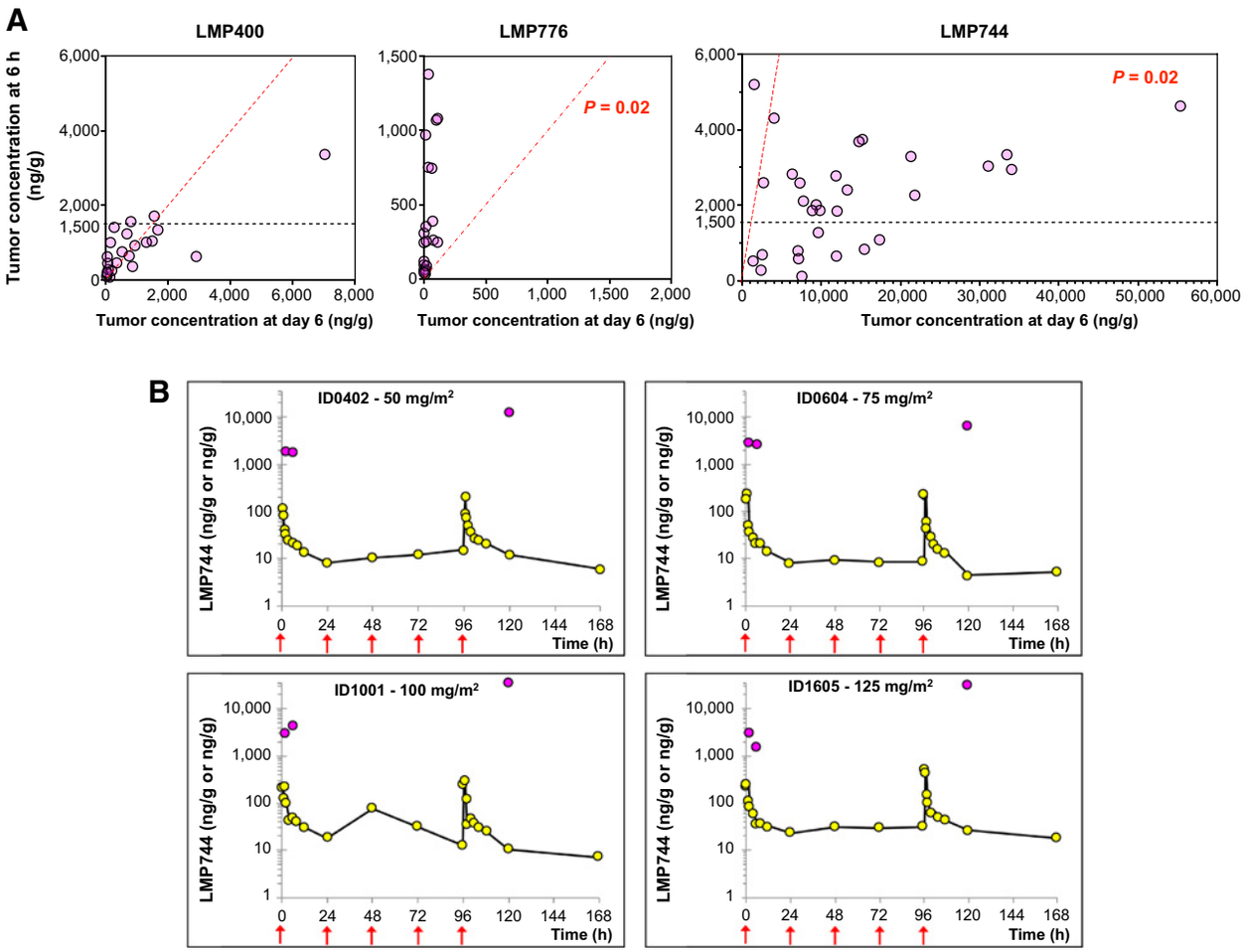


Figure 3.

Tumor accumulation/retention of LMP744 (right) compared with the two other indenoisoquinolines (see Supplementary Tables S4–S6 for detailed data). **A,** Red dashed lines denote equivalency; points to the right of the line reflect drug accumulation [day 6/day 1 (6 hours)]. Statistical significance determined for relationship between indenoisoquinoline levels 6 hours after the first drug dose and 24 hours after the last drug dose (6 days). **B,** Plasma (●) and tumor (●) levels of LMP744 in representative animals at four dose levels. Full pharmacokinetic sampling was performed after the first and fifth doses and trough levels were obtained prior to dosing on days 2 to 5.

LMP400 exhibited significant activity despite the fact dose escalation was not intended to reach MTD, because MTD was determined in a prior human study (11). Notably, LMP744 showed substantially greater efficacy than the two other indenoisoquinolines; even in the dose escalation cohort, clear evidence of activity was demonstrated, with one dog experiencing a complete remission at dose level 2 (50 mg/m^2). The LMP744 dose expansion cohort confirmed responses at the MTD in a larger group of animals ($n = 12$) along with demonstration of drug tolerability at this dose level. The majority of the clinical responses seen were partial in nature, with best response typically observed within 14 days of drug administration. In light of the substantive therapeutic efficacy of LMP744 at dose levels well below the MTD, it is possible that this agent could be considered for further study as part of a combination strategy with other DNA-damaging agents or agents that modify the DNA damage response (19, 20).

The notable activity of LMP744 at all dose levels is likely due to its unique pharmacokinetics. Indeed, despite the apparently limited chemical differences between the three indenoisoquinolines tested here (see Fig. 1), LMP744 demonstrated an extended half-life (17 hours), a high volume of distribution (1470 L/m^2), and high tumor retention and accumulation, yielding drug concentrations in the tumor much higher one day after the last dose than after its first administration. Furthermore, using two validated pharmacodynamic biomarkers, histone γH2AX and TOP1 protein levels (11, 12, 14–16, 18, 28), target engagement and a DNA damage response was demonstrated for all three indenoisoquinolines examined in our trial. On the basis of this study, LMP744 appears to be a potentially promising candidate for human clinical trials. The fact that low doses of LMP744 with minimal bone marrow toxicity produced significant activity in canine lymphoma led to the development of a currently active phase I trial at the NIH Clinical Center (NCT03030417).

In conclusion, this comparative oncology study enabled a robust, biologically rich evaluation of the efficacy, toxicity, pharmacokinetics, and mechanism of action of three novel

indenoisoquinolines (LMP776, LMP400, and LMP744) in spontaneous canine lymphoma. It validated two pharmacodynamic biomarkers for evaluating this class of agents during clinical trials, and provided important insights into their potential mechanisms of action in spontaneous non-Hodgkin lymphoma. Furthermore, use of a multicenter consortium, such as the NCI-COTC, demonstrated the ability of a veterinary consortium to rapidly dose-escalate and randomize multiple candidates in a drug class, while simultaneously collecting high-quality biologic and clinical datasets that have been highly informative for human phase I IND filings, the development of biomarkers of the DNA damage response, and the design of human early-phase trials. The efficacy signal observed for dogs with peripheral lymphomas, coupled with facile access to biologic samples, will aid in prioritizing these agents for advancement to human efficacy and proof-of-concept testing in the phase II setting.

Disclosure of Potential Conflicts of Interest

C. Khanna is an employee of and has ownership interests (including patents) at Animal Clinical Investigation. No potential conflicts of interest were disclosed by the other authors.

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