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Phase 1 Dose-Escalation Study with LEC/chTNT-3 and Toceranib Phosphate (Palladia®) in Dogs with Spontaneous Malignancies

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Abstract

Objectives: LEC chemokine promotes TH1 responses and recruits immune cells to inflammatory sites. By linking LEC to an antibody targeting tumor necrosis, LEC/chTNT-3 can be used for the immunotherapeutic treatment of tumors. The primary objective of this study was to determine the safety profile of LEC/chTNT-3 and toceranib phosphate (Palladia®) combination therapy in dogs with spontaneous malignancies. Secondary purpose was to determine objective responses to treatment.

Methods: Twenty-three dogs with cancer were enrolled, covering nine different malignancies. In this dose escalation study, dogs received LEC/chTNT-3 for five days, and toceranib every 48 hours for the remainder of the study. Dogs received physical exams, chemistry panel, urinalysis, and complete blood counts on days 0, 10, 28 of the study, and every 6-8 weeks thereafter.

Results: Lethargy was noted in 13% dogs. There were no statistical differences in the prevalence of anorexia, diarrhea, thrombocytopenia, renal toxicity, or hepatic toxicity before or during the study. There were trends in increases in the prevalence of vomiting, lymphopenia, and neutropenia (all grade 2 or lower, p=0.07) over the initial 28 days of the study. By day 28, 10% of dogs had partial responses, 58% had stable disease, and 32% had progressive disease.

Conclusions: LEC/chTNT-3 and toceranib were well tolerated. This combination therapy showed some biological activity against a variety of cancers at a low dose and short duration of LEC/chTNT-3 administration.

Keywords: Immunotherapy; LEC; TNT antibody; Toceranib; Canine; Safety; Chemokine; Immunocytokine

Introduction

Liver Expressed Chemokine (LEC, CCL16) recruits immune cells to tumors and promotes anti-tumor responses [1-6]. Dendritic cells and macrophages, as well as other cell types, naturally secrete LEC to recruit lymphocytes and monocytes to inflammatory sites [7] through its receptors CCR1, CCR2, and CCR5 [8]. In addition to its chemotactic activity, LEC can modulate immune function by suppressing myeloid progenitor cell proliferation [7], while promoting antigen-presenting functions of macrophages and dendritic cells and enhancing T cell cytotoxicity [9,10]. LEC increases the production of T helper type 1 (T_H1) cytokines [9,10] and enhances the killing activity of macrophages and cytotoxic T cells through increased Fas ligand, perforin, and granzyme B expression [9,11]. Several groups demonstrated that human LEC reduces tumor burden, and promotes tumor infiltration by immune cells in murine tumor models [1-6,11]. In mice bearing Colon 26 tumors, tumors completely regressed when combining LEC/chTNT-3 therapy with depletion of T regulatory cells, and was accompanied by protection against tumor rechallenge, indicating that a protective immune memory response was achieved [4]. With the exception of a tumor-infiltrating lymphocyte (TIL) therapy protocol, tumor studies using LEC required the presence of LEC at the tumor site to demonstrate efficacy [1-5]. For research purposes, other groups achieved localization of LEC to tumors through adenoviral delivery [1-3]. As an alternative to viral delivery which is not easily translated into clinical use, we developed a LEC fusion protein in which human LEC is genetically fused to tumor-targeting chimeric antibody TNT-3 (chTNT-3) [4-6,12].

Previous work demonstrated that solid tumors could be targeted using monoclonal antibodies, designated Tumor Necrosis Therapy (TNT) [13-20], directed against ubiquitous and stable nucleic acid antigens retained in necrotic tissues. Since necrosis is a universal feature of solid tumors and is increased by cytoreductive therapies, TNT antibodies have a number of advantages over other targeting approaches [18]. One important advantage is that TNT antibodies target antigens that are not specific to a single species, as demonstrated in previous human and mouse studies [4,5,13-16,18-23], and therefore, can be used in both experimental animal models and clinical settings for humans. Human clinical trials using TNT antibodies for radiotherapy of recurrent solid tumors, including lung carcinomas and brain cancers, showed that TNT antibodies specifically target tumors in patients with limited binding of normal tissues [13,15,20]. Similarly, we previously established the tumor-targeting specificity of these antibodies and their derivatives, including chTNT-3, in imaging studies, [16,18,19] and demonstrated their therapeutic potential as antibody conjugates or fusion proteins in murine tumor models [4,5,14,21-23]. This present work is the first study of chTNT-3 in dogs.

Because chTNT-3 can bind areas of necrosis in multiple species and human LEC has shown therapeutic effects in mouse tumor

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models, we applied LEC/chTNT-3 in a preclinical canine cancer study treating nine different types of cancers. Previous studies in mice showed that deletion of suppressor cell types enhanced the therapeutic effect of LEC/chTNT-3 [4]. Therefore, toceranib phosphate (Palladia®, Pfizer, Madison, NJ), a receptor tyrosine kinase inhibitor related to sunitinib, was added to the study as a method to suppress myeloid derived suppressor cells (MDSC) and T regulatory cells [24,25]. In addition to its immunomodulatory effects, toceranib was originally developed as an anti-angiogenic agent and has been shown to inhibit oncogenic tyrosine kinases, such as KIT and RET [26]. As one of the two FDA approved canine-specific anti-neoplastic drugs, toceranib is indicated for the treatment of mast cell tumors and is generally used in conjunction with other modes of therapy, although trials have shown modest clinical benefits in other types of tumors [27-30].

The main objective of this dose-escalation study was to determine the safety profile of LEC/chTNT-3 in conjunction with toceranib. In addition to establishing its safety, we suggest an optimal dosing range of LEC/chTNT-3 for efficacy in dogs with advanced stages of malignancies.

Materials and Methods

Design of clinical study

Dogs with established cases of cancer were recruited from the Veterinary Centers of America West Los Angeles Animal Hospital (Los Angeles, CA), under a protocol approved by the clinic's Institutional Animal Care and Use Committee (IACUC). Owners were required to read and sign an informed consent prior to enrollment. Pre-enrollment biopsy or cytology, full blood analysis, urinalysis, and diagnostic imaging were performed to confirm and stage tumors. Starting on day 1 of the study, owners administered to patients five daily subcutaneous injections of LEC/chTNT-3, ranging in dose from 17.8-540 μg/kg body weight, in the lateral stifle, intrascapular, hind quarters, flank, or thorax areas depending on owners' preferences. In this dose escalation study, the initial cohort of patients received 17.8-25 μg/kg LEC/chTNT-3. If no toxicities were noted, the subsequent cohort received up to 50 μg/kg LEC/chTNT-3. Cohort 3 received up to 75 μg/kg LEC/chTNT-3, cohort 4 up to 250 μg/kg, and cohort 5 up to 540 μg/kg. Concurrent to LEC/chTNT administration on day 1, patients received toceranib orally once every 48 hours with food at doses, 2.1-2.8 mg/kg. This dose is considered sufficient for target inhibition, but has been shown to result in a reduced adverse event profile from that associated with the standard dose, 3.25 mg/kg [31].

Patients were seen on days 0, 10 and 28 of the study for a full physical exam, blood analysis, urinalysis, and imaging, if indicated. After the initial 28 days, patients were seen once every 6-8 weeks for full physical exams and imaging. If clinical response had been achieved by day 28 of the study with no significant toxicities, patients continued receiving toceranib indefinitely while still enrolled in this study. If the tumor was determined to be progressive the patient was eligible to then undergo alternate therapies. Owners were allowed to remove their dogs from the study at any time. Decision to euthanize a given patient was done at the discretion of the owners with recommendations from the veterinarians.

Toxicity and response criteria

Symptoms of toxicity, such as lethargy, anorexia, diarrhea, and vomiting, were reported by owners over the initial 28 days of the study. Urinalysis, chemistry blood panel, and complete blood count were taken on days 0, 10 and 28 of the study to assess for hematologic, metabolic, renal, and liver toxicities. Grading of the toxicities followed the criteria for grading adverse events following chemotherapy and anti-neoplastic biologics published by the Veterinary Cooperative Oncology Group (VCOG) [32]. Similarly, response to treatment was determined using imaging modalities and caliper measurements. Tumors, including metastasis, were measured for a minimum of 2 dimensions, which were added for an overall tumor size. Progressive disease is defined as having >25% progression in any existing lesion (including metastatic lesions) or having new lesions; stable disease as having <50% resolution and <25% progression in existing lesions, and no new lesions; and partial response as having >50% resolution and no new lesions.

Generation of LEC/chTNT-3

The C-terminus of LEC was genetically linked to the N-terminus of the chTNT-3 heavy chain with a 5 amino acid universal linker (Gly_4Ser), yielding 2 LEC molecules per antibody. The construction, production, and purification of LEC/chTNT-3 have been previously described in detail [5]. After purification, chTNT-3/LEC in phosphate-buffered saline was passed through a 0.22 μm sterilization filter (EMD Millipore, Billerica, MA) and vialed prior to storage and administration to patients.

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) canine melanoma tissue sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval (0.1 M citrate, pH 6.0) and incubated with 3% H_2O_2 to block endogenous peroxidase activity. Because chTNT-3 does not bind to FFPE sections, chTNT-1 was used as a surrogate. Sections were stained overnight at 4°C with chTNT-1 (1 μg/mL) in 2.5% goat serum or 2.5% goat serum alone as a negative control. Antibody detection was done using 3,3′-diaminobenzidine and VECTASTAIN ABC Kit Human IgG (Vector Laboratories, Burlingame, CA) according to manufacturer's protocol. Images were captured on a Leitz Orthoplan microscope using a Nikon DS-Fi2 camera.

Migration assay

Canine monocytes were isolated by differential density centrifugation (Ficoll Hypaque, Sigma, St. Louis, MO) of peripheral blood from four canine patients and cryopreserved. Freshly thawed cells were used in each migration assay. Cells were stained in 5 μg/mL calcein-AM (Life Technologies, Carlsbad, CA) in RPMI-1640 at 37°C for 30 minutes prior to the assay. Migration was assessed using 96-well microchemotaxis plates and filters with 5 μm pores (ChemoTx System, Neuro Probe, Gaithersburg, MD). LEC/chTNT-3, parental chTNT-3, or PBS were diluted in binding medium (1% BSA/RPMI-1640) and placed in the lower chamber of the chemotaxis plate. Five x 10^4 cells were added to the top chamber in binding medium and allowed to migrate for 1.5 hours at 37°C in a humidified incubator. Non-migrated cells were removed by rinsing the top of the chambers with PBS and wiping with a cell scraper. Migrated cells were quantified using a BioTek Synergy HT fluorescence plate reader (BioTek Instruments, Vermont, USA) (excitation 485 nm/emission 528 nm). Fluorescent units were converted into cell numbers using a standard curve of stained cells. Migration ratios represent the number of migrated cells to LEC/chTNT-3 or chTNT-3 divided by the number of cells exposed to PBS. All assays were done in duplicate.

Statistical analysis

Statistical analysis was completed using GraphPad Prism 6 (La

Jolla, CA) and JMP Pro 11.0.0 (SAS Institute, Cary, NC). A two-tailed independent t-test was used to compare migration ratios between chTNT-3 and LEC/chTNT-3. For toxicities, the prevalence of toxicity prior to LEC/chTNT-3 administration was compared to prevalence of toxicity reported during the initial 28 days of the study using a Chi-square test. A log-rank test was used to analyze effect of dose on progression-free survival and overall survival, and a Cox proportional hazard model was used to demonstrate a dose response on overall survival.

Results

Canine activity of TNT antibody and LEC/chTNT-3 *in vitro*

TNT antibodies bind to nucleic acid structures in the nucleus that become accessible in necrotic regions of tumors [13-20]. In addition to its reactivity in murine and human cells, its binding to canine tissues was demonstrated in paraffin-embedded tissues, where TNT antibody bound to nuclei (Figure 1A). To demonstrate the activity of the LEC moiety on the migration of canine mononuclear cells, LEC/chTNT-3 was compared to parental chTNT-3. Cells exposed to chTNT-3 did not demonstrate directed migration. However, there was a statistically significant increase in migration with exposure to LEC/chTNT-3 (p=0.01, Figure 1B), verifying the chemotactic activity of the human LEC/chTNT-3 on canine immune cells *in vitro*.

Patients' characteristics

This study included patients from at least 15 breeds, ranging in age from 6-18 years old (mean 10.5 years). Of the 23 dogs enrolled, 2 were intact males, 10 were spayed females, and 11 were neutered males. This report included cases of hepatocellular carcinoma (2 cases), melanoma (7), soft tissue sarcoma (6), squamous cell carcinoma (4), hepatic sarcoma (1), apocrine gland carcinoma (1), mast cell tumor (1), osteosarcoma (1), and histiocytic sarcoma (1). Time from diagnosis to enrollment in the study ranged from 1 week to 15 months (mean 6.1 months, median 5 months). With the exception of three cases, tumors were previously treated with surgery, radiation therapy, and/or chemotherapy (including toceranib), and were refractory to previous therapy. For feasibility reasons, subcutaneous injections were chosen as the route of administering LEC/chTNT-3 to allow pet owners to administer treatment. Upon enrollment, patients received 5 daily subcutaneous injections of LEC/chTNT-3, ranging in dose from 17.8-540 µg/kg body weight, and toceranib orally every other day. Patients' characteristics pertaining to the study along with LEC/chTNT-3 dosing are listed in Table 1.

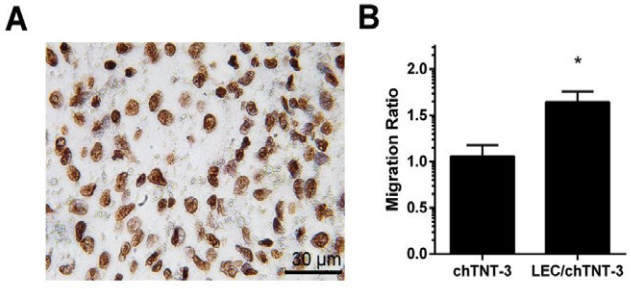

Figure 1: *In vitro* activity of TNT antibody and LEC/chTNT-3.
A) FFPE canine melanoma section stained with TNT antibody (400x magnification). B) Chemotactic activity of 100 nM LEC/chTNT-3 and parental chTNT-3 on canine mononuclear cells. Ratios represent the number of cells migrated exposed to LEC/chTNT-3 or chTNT-3 compared to cells exposed to PBS (random migration). Error bars represent SEM. Samples were ran in duplicate. N=4, *p=0.01

Safety and potential toxicities

LEC/chTNT-3 and toceranib were well tolerated throughout the study. During days 1-5, when LEC/chTNT-3 was being administered, no new onsets of or worsening nausea, diarrhea, or anorexia were reported. Three dogs (13%) were described as lethargic during one of the five days of treatment administration, while one dog was described as having increased energy and appetite. In one case, an owner noted stiffness in the dog's gait lasting four hours after intrascapular injection on day 3, which was attributed to sensitivity at the injections site.

Patients received a physical exam, full blood analysis, urinalysis, and diagnostic imaging on days 10 and days 28. Periodic lethargy over this time period was reported by owners in three of the patients (13%). Individual cases of yeast otitis, bacterial rhinitis, and urinary tract infection occurred during this time, but were not attributed to LEC/chTNT-3 or toceranib and were quickly resolved. Weight loss, fever, gastrointestinal side effects, hematological, renal, and hepatic toxicities are summarized in Table 2. Prevalence of toxicities over 28 days following LEC/chTNT-3 administration was not statistically different from prevalence prior to treatment; however, there was a trend that the prevalence of vomiting (16.7%) was greater during than prior to the study (p=0.07). New or worsening incidences of vomiting, diarrhea, and anorexia did not correlate with escalating doses of LEC/chTNT-3 (Figure 2A). While not statistically significant, there was a trend that the prevalence of neutropenia and lymphopenia (p=0.07) were greater after treatment with LEC/chTNT-3 (Table 2). This trend is also observed when analyzing new or worsening cases of neutropenia and lymphopenia in their relation to escalating doses of LEC/chTNT-3 (Figure 2B). All hematologic toxicities were grade 1 or 2, and neutropenia is a known side effect of toceranib [28,31]. However, neutropenia and lymphopenia could also be due to recruitment of leukocytes to the tumor site [4,5,33], resulting in lower observed circulating leukocyte counts.

Similar to conventional therapies, such as radiotherapy or chemotherapy, LEC/chTNT-3 immunotherapy is expected to cause necrosis in cancer tissue. While tissue necrosis is a sign of treatment efficacy, rapid necrosis can cause unsightly loss of tumor tissue located at the body surface, and more critical complications depending on the tumor site(s). In one case of squamous cell carcinoma located in the nasal planum, loss of the nasal planum tissue was noted (patient 1). Ulcerations of externally-located tumor tissue was observed in three other patients (patients 5, 11, and 23), and was the reason for the euthanasia of three of the dogs (patients 1, 11, and 23). Out of the 23 patients treated, two had complications arising from the rapid necrosis of cancer tissue, which were evident in the gross pathology during necropsy. One dog with metastatic melanoma (patient 14) was euthanized after developing a pneumothorax secondary to necrosis of lung metastasis 32 days after receiving LEC/chTNT-3 and toceranib. Another dog with metastatic melanoma (patient 5) passed away 9 days after receiving therapy due to necrosis of a cardiac metastasis that was only evident after necropsy. While such complications can be expected with treatment of tumors in vital organs, these examples underscore limitations of treatment and possible contraindications in the design of future clinical trials.

Survival and response

Of the 23 patients, 18 (78%) were in late-stages of disease, and all

	LEC dose (µg/kg)	Tumor[a]	Stage	Time from dx to LEC treatment	Previous therapy[b]	Treatment following LEC and toceranib[c]	Survival post-LEC (months)	Status
1	17.8	scc	III	1 yr	chemo	-	1.2	euthanized
2	21.2	hepatic sarcoma	III	1 wk	-	-	2.8	euthanized
3	22.2	digital melanoma	III	8 mo	chemo, toceranib, vax	carboplatin, temozolamide	4.7	euthanized
4	22.5	soft tissue sarcoma	Ib	5 mo	XRT, chemo, piroxicam	-	2.1	euthanized
5	25.0	oral melanoma	IV	2 mo	-	-	0.3	died
6	33.3	soft tissue sarcoma	IIa	7 mo	chemo	CTX	4.7	died
7	40.0	hcc	III	1 mo	chemo	mitoxantrone	9.9	euthanized
8	42.5	oral melanoma	IV	7 mo	chemo, vax	CTX	9.5	euthanized
9	44.1	hcc	III	7 mo	surgery	-	18.2	euthanized
10	45.0	oral melanoma	IV	14 mo	chemo, vax	XRT	3.8	died
11	67.0	scc	II	5 mo	XRT	-	0.5	euthanized
12	67.5	scc	I	3 mo	chemo, piroxicam	bleomyocin	31.1	alive
13	69.0	soft tissue sarcoma	Ib	1 wk	-	surgery, XRT	2.5	alive
14	70.0	digital melanoma	III	8 mo	surgery, XRT	-	3.0	euthanized
15	70.0	scc	III	1 mo	piroxicam	-	3.7	alive
16	82.8	oral melanoma	III	5 mo	chemo, toceranib, vax	-	1.7	died
17	103	osteosarcoma	IIb	4 mo	chemo	-	5.4	euthanized
18	133	apocrine gland carcinoma	III	7 mo	XRT, chemo, toceranib	XRT	17.9	died
19	211	histiocytic sarcoma	IIIb	9 mo	chemo	prednisone	2.2	euthanized
20	330	oral melanoma	IV	1 mo	XRT	-	2.1	euthanized
21	524	soft tissue sarcoma	IIb	1 yr	surgery	prednisone	1.1	euthanized
22	540	soft tissue sarcoma	Ib	5 mo	surgery, XRT	doxorubicin	6.3	alive
23	540	soft tissue sarcoma, mast cell tumor	IIb, III	15 mo, 5 yr	surgery, chemo, toceranib	-	0.7	euthanized

[a]scc = squamous cell carcinoma, hcc = hepatocellular carcinoma [b]XRT = radiation, chemo = chemotherapy, vax = vaccine (ONCEPT®) [c]CTX = cyclophosphamide

Table 1: Patient characteristics.

	Day 0	Day 10	Day 28	
Weight Changes from Day 0				
5-7.5% weight loss	-	13.6% (3/22)	0% (0/21)	
7.5-10%	-	0% (0/22)	11.1% (2/18)	
> 10%	-	0% (0/22)	5.6% (1/18)	
total	-	13.6% (3/22)	16.7% (3/18)	-
Fever (≥ 103°F)	0% (0/20)	5.6% (1/18)	5.6% (1/18)	p = 0.26
GI Toxicities				
Anorexia	17.4% (4/23)	27.3 % (6/22)	11.1% (2/18)	p = 0.30
Grade 1-2	3/23	4/22	0/18	
Grade 3-4	1/23	2/22	2/18	
Vomiting	0% (0/23)	0% (0/22)	16.7% (3/18)	p = 0.07
Grade 1-2	0/23	0/22	3/18	
Grade 3-4	0/23	0/22	0/18	
Diarrhea	8.7% (2/23)	4.5% (1/22)	11.1% (2/18)	p = 0.64
Grade 1-2	2/23	1/22	2/18	
Grade 3-4	0/23	0/22	0/18	
Hematologic Toxicities (all grade ≤ 2)				
Neutropenia	0% (0/23)	9.1% (2/22)	11.8% (2/17)	p = 0.07
Thrombocytopenia	0% (0/23)	4.5% (1/22)	0% (0/17)	p = 0.31
Lymphopenia	0% (0/23)	13.6% (3/22)	11.8% (2/17)	p = 0.07
Renal Toxicity (Creatinine) (all grade ≤ 2)	4.3% (1/23)	4.5% (1/22)	0% (0/17)	p = 1.0
Hepatic Toxicity (ALT)	17.4% (4/23)	18.2% (4/22)	17.6% (3/17)	p = 0.71
Grade 1-2	3/23	3/22	2/17	
Grade 3-4	1/23	1/22	1/17	

Table 2: Toxicities.

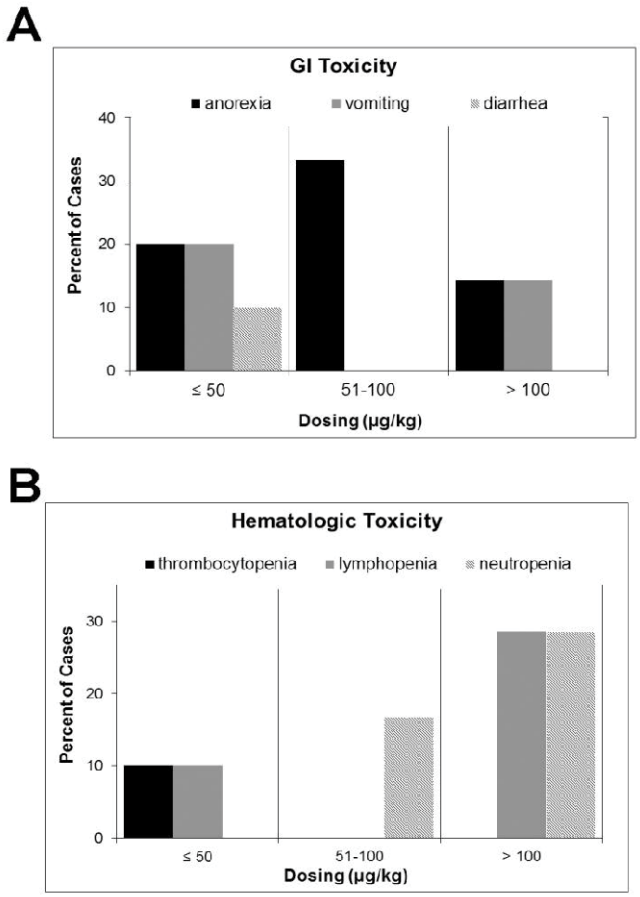

Figure 2: Relationship between toxicities and dose of LEC/chTNT-3. A) Incidences of new or worsening cases of gastrointestinal toxicities during the initial 28 days of the study are not dependent on LEC/chTNT-3 dose. B) Incidences of new or worsening cases of neutropenia (all grade ≤ 2) showed a trend of dose dependency (p=0.08). Trends for dose dependency were analyzed using the chi-square test for trend.

had progressive diseases. To illustrate the range in tumor presentation, images from three cases (melanoma, squamous cell carcinoma, and histiocytic sarcoma, respectively) are shown in Figure 3. Necrosis of the visible tumor is apparent in the dark discoloration seen within four days of initiating LEC/chTNT-3 and toceranib therapy (Figure 3A). Similar results were seen in other patients with ulcerated tumors. In Figures 3B and 3C, resolution of the main tumor mass and reduction in the number of tumor nodules are demonstrated within a month of initiating LEC/chTNT-3 and toceranib therapy.

Twenty-two of the 23 patients (96%) received some clinical benefit, as defined by having stable disease or a partial response, by day 10 (Figure 4A). The clinical benefits were retained in 13 of the patients for at least three weeks after the last administration of LEC/chTNT-3. These results are encouraging considering the advanced stages of presentation and the wide range in dosage of LEC/chTNT-3. Average duration of response or clinical benefit (SD or PR) was 67 days (median 40 days).

While not statistically significant, Kaplan-Meier analysis showed trends of improved progression-free survival (Figure 4B, p=0.08) and survival post-LEC/chTNT-3 therapy (Figure 4C, p=0.2) in the two cohorts receiving 26-75 µg/kg LEC/chTNT-3. The Cox proportional hazards model demonstrated a hazards ratio of 0.94 (95% CI: 0.88-0.98, p=0.01) with increasing doses of LEC/chTNT-3, up to 75 µg/kg. For every unit (µg/kg) increase in LEC/chTNT-3 dose, up to 75 µg/kg, there was a 5% reduction in death in these canine cancer patients. Interestingly, with doses above 75 µg/kg, the proportional hazards model did not demonstrate improved overall survival with increasing dosage (p>0.05). While this study is a preliminary investigation of the safety and therapeutic profile of short-term administration of LEC/chTNT-3 combined with toceranib in dogs with a varied history and presentation of cancer, we provide a suggested dose range for future clinical trials.

Discussion

In this dose escalation study, up to 540 µg/kg LEC/chTNT-3 was administered daily for five consecutive days concurrently with oral toceranib (2.1-2.8 mg/kg) every other day for the remainder of the study. The treatment regimen was well tolerated. In cases where tumors were visibly exposed, rapid necrosis of the tumor tissue could be seen within days of initiating LEC/chTNT-3 and toceranib therapy. These observations are noteworthy, because while human LEC has been shown to have biological activity in mice [1-5] and chTNT-3 alone has no therapeutic effect [4,5,14,22,23], to our knowledge, this is the first

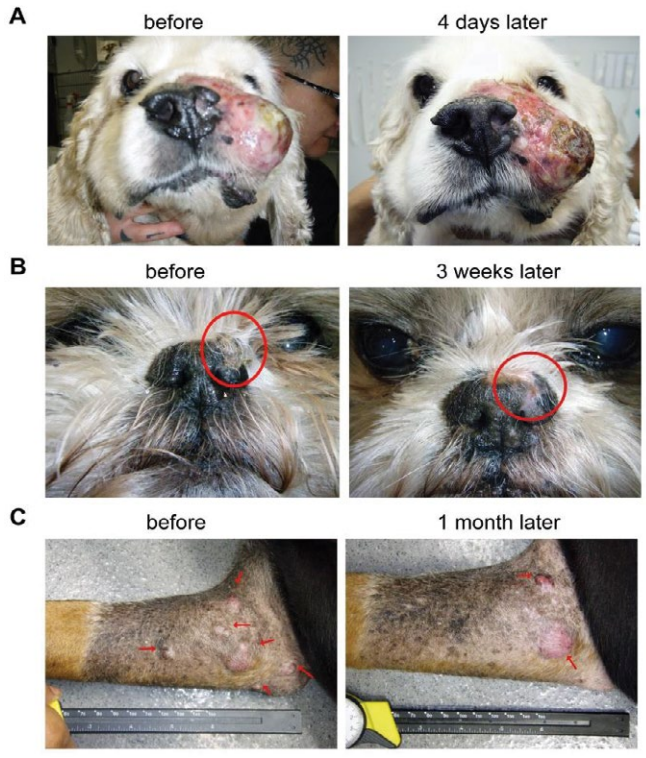

Figure 3: Images before and after treatment with LEC/chTNT-3 and toceranib phosphate. A) Patient 5 from Table 1 with stage IV oral melanoma showing ulcerated tumor. Main tumor mass is 70 cm^3 before treatment, and 31 cm^3 after treatment with LEC/chTNT-3. B) Patient 15 from Table 1 with stage III squamous cell carcinoma. Tumor is denoted by the red circles. After 3 weeks, over 90% of the tumor mass has been resolved. Residual tumor and scar tissue can be seen by the white tissue on the black nasal planum. C) Patient 19 from Table 1 with stage IIIb histiocytic sarcoma. The right thoracic limb is shown. Tumor nodules are indicated by the red arrows. Complete resolution of 4 of the 6 nodules following treatment can be seen in these images.

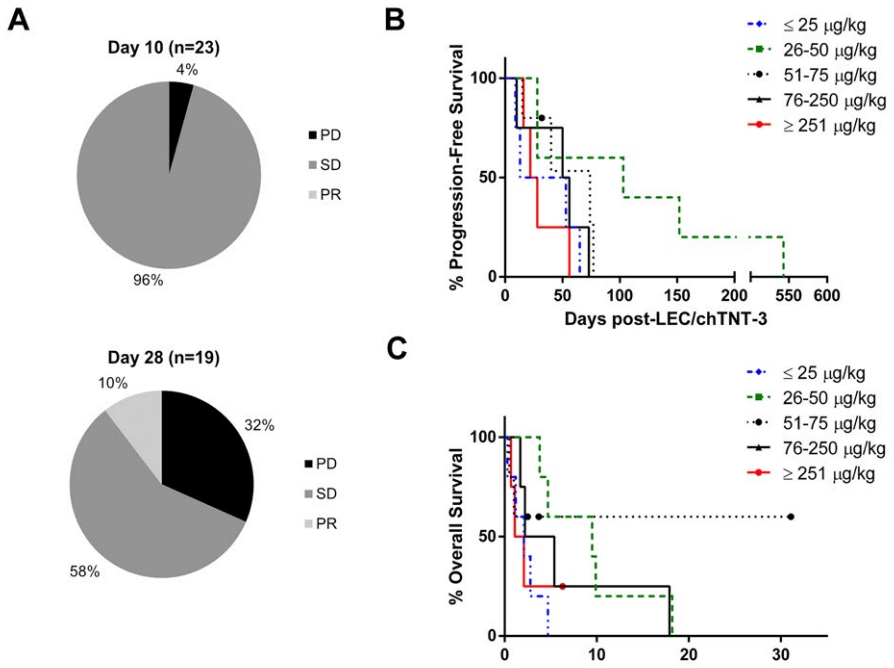

Figure 4: Clinical response of LEC/chTNT-3 and toceranib phosphate.
A) Pie charts illustrating clinical benefit (defined as being characterized by stable disease and partial response) by day 10 and day 28 of the study. B) Kaplan-Meier curve showing progression-free survival based on dosing cohorts. p=0.08 as determined by a log-rank test. C) Kaplan-Meier curve showing effect of dosing cohorts on survival. P=0.2 as determined by a log-rank test.

time human LEC has been suggested to have activity in dogs. However, contributing effects from toceranib cannot be ruled out.

Because of the late stage of disease in many cases, complications did arise from the rapid necrosis occurring in metastasis in the heart and lungs of two dogs. Similarly, the necrosis that occurred in visibly exposed tumors led to deteriorating appearances that contributed to owners deciding to euthanize their dogs. However, it should be noted that in two cases, the dogs came into the study with ulcerative tumors or a tumor so extensive that the entire nasal planum was absent. Decisions of when to euthanize patients were ultimately in the hands of the owners, reflecting personal and economic factors, and highlight a difficulty of conducting clinical trials in terminally ill dogs. As opposed to human clinical trials or murine tumor studies, survival data in canine cancer therapy trials are confounded by the fact that decisions to euthanize patients are not based on objective or predefined criteria. For this reason, many phase I trials in dogs with advanced diseases do not report survival data, and we did not compare our survival with average prognosis for each tumor type. However, we did compare survival of the dosing cohorts to see if there was any preliminary indication of an optimal dosing range for LEC/chTNT-3. Based on current status, clinical response, and survival post-LEC/chTNT-3, 40-75 µg/kg LEC/chTNT-3 per dose (Table 1 and Figure 4) is suggested to provide optimal effect. Similarly, in previous murine studies, response to LEC/chTNT-3 was dose responsive up to an optimal dose, with a decrease in therapeutic effect at higher doses (> 2.5 µg/g body weight) (unpublished data).

In previous murine studies with LEC, complete cures of tumors were only achieved when T regulatory cells were depleted [4] or antagonistic antibodies to IL-10R were added to the therapy [3]. These observations demonstrate that, like in most other treatments, inhibition of immunosuppressive cell types need to be a key component of successful immunotherapy. Because tyrosine kinase inhibitor sunitinib has been shown to reduce MDSC [34] and T regulatory cells [35] in human cancer patients and suppressor cell types in tumor-bearing animals [25], we included a structurally similar tyrosine kinase inhibitor, toceranib phosphate, that has been approved as adjunctive therapy for canine mast cell tumors to be used in combination with LEC/chTNT-3. It should be noted that while elevated MDSC have been characterized in dogs with cancer [36,37], no study has investigated whether toceranib actually lowers canine MDSC levels *in vivo*. It will be important for future immunotherapy studies to measure circulating MDSC, T regulatory cells, and immune effector cells in canine patients to assess possible limitations on efficacy.

Clinical toxicities associated with toceranib are primarily gastrointestinal side effects and neutropenia [27-31,38]. While lethargy was reported in 13% (3/23) of dogs during the 5 days of administration of LEC/chTNT-3, no gastrointestinal side effects were noted during this time. Lethargy seen in these cases may be due to cytokine release which can be measured in subsequent clinical trials. In the initial 28 days of the study, incidences of other toxicities were seen, most notably vomiting, mild neutropenia, and mild lymphopenia, and may be due to LEC/chTNT-3, toceranib, the combination of LEC/chTNT-3 and toceranib, or the advanced stage of disease. While neutropenia is an expected side effect of toceranib, lower circulating leukocyte counts also may be expected with greater recruitment of immune cells to tumor sites [1-5].

While determining the safety profile of combination LEC/chTNT-3 and toceranib treatment was the primary objective of this phase I

trial, preliminary data suggested a biological response to combination therapy. Preliminary evidence of biologic activity of toceranib alone or in combination with metronomic NSAIDs or cyclophosphamide has been demonstrated in non-mast cell tumors with clinical benefit (complete response, partial response, or stable disease) reported in 54-77% of dogs [28,29,31]. In our study, clinical benefit was noted in 68% of dogs over 28 days. However, it should be noted that the previously quoted studies enrolled cancer patients with life expectancies greater than 6-12 weeks, and only two dogs were euthanized in those studies [28,29,31]. In contrast, the heterogeneity in clinical presentation and advanced stages of our patients were limitations in our study design. Any dog with gross oncological disease could have been enrolled in this trial. In our study, 48% (11/23) of the dogs were euthanized or died within 3 months of receiving treatment, reflecting their advanced stages. While the contribution of toceranib to the clinical benefits cannot be ruled out, LEC/chTNT-3 is likely to have played a principal role in the observed biological responses. Three of the dogs in this study were previously receiving toceranib in addition to chemotherapy and surgery, radiation therapy, or vaccine therapy, and all were refractory to previous therapy (Table 1). While responses continued for several weeks after receiving LEC/chTNT-3, toceranib was still being administered after the duration of response was observed to have ceased. In other words, of the dogs showing initial responses to LEC/chTNT-3 and toceranib combination at day 28, 77% (10/13) of dogs eventually had progressive disease even though they were still receiving toceranib.

Because LEC/chTNT-3 and toceranib therapy were well tolerated, it may be beneficial to add subsequent cycles of LEC/chTNT-3 in future trials. These future trials should analyze tumor-infiltrating immune cells to characterize and confirm the biologic responses to LEC/chTNT-3. In addition, the measurement of MDSC and T regulatory cells using flow cytometry, and serum cytokine measurements would provide added insight into the efficacy and limitations of combination LEC/chTNT-3 and toceranib therapy. In adding subsequent cycles of LEC/chTNT-3 or lengthening the treatment period, trials will need to monitor signs of autoimmunity, as autoimmunity was not seen in this short trial. While this study is a pilot investigation of the safety and therapeutic profile of short-term administration of LEC/chTNT-3 and toceranib in dogs with a varied history and presentation of cancer, this study serves as a preliminary work for LEC/chTNT-3 immunotherapy in future clinical trials.

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References

1. Giovarelli M, Cappello P, Forni G, Salcedo T, Moore PA, et al. (2000) Tumor rejection and immune memory elicited by locally released LEC chemokine are associated with an impressive recruitment of APCs, lymphocytes, and granulocytes. J Immunol 164: 3200-3206.
2. Guiducci C, Di Carlo E, Parenza M, Hitt M, Giovarelli M, et al. (2004) Intralesional injection of adenovirus encoding CC chemokine ligand 16 inhibits mammary tumor growth and prevents metastatic-induced death after surgical removal of the treated primary tumor. J Immunol 172: 4026-4036.
3. Guiducci C, Vicari AP, Sangaletti S, Trinchieri G, Colombo MP (2005) Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res 65: 3437-3446.
4. Li J, Hu P, Khawli LA, Epstein AL (2003) Complete regression of experimental solid tumors by combination LEC/chTNT-3 immunotherapy and CD25(+) T-cell depletion. Cancer Res 63: 8384-8392.
5. Li J, Hu P, Khawli LA, Epstein AL (2003) LEC/chTNT-3 fusion protein for the immunotherapy of experimental solid tumors. J Immunother 26: 320-331.
6. Lechner MG, Karimi SS, Barry-Holson K, Angell TE, Murphy KA, et al. (2013) Immunogenicity of murine solid tumor models as a defining feature of in vivo behavior and response to immunotherapy. J Immunother 36: 477-489.
7. Youn BS, Zhang S, Broxmeyer HE, Antol K, Fraser MJ Jr, et al. (1998) Isolation and characterization of LMC, a novel lymphocyte and monocyte chemoattractant human CC chemokine, with myelosuppressive activity. Biochem Biophys Res Commun 247: 217-222.
8. Nomiyama H, Hieshima K, Nakayama T, Sakaguchi T, Fujisawa R, et al. (2001) Human CC chemokine liver-expressed chemokine/CCL16 is a functional ligand for CCR, CCR2 and CCR5, and constitutively expressed by hepatocytes. Int Immunol 13: 1021-1029.
9. Cappello P, Caorsi C, Bosticardo M, De Angelis S, Novelli F, et al. (2004) CCL16/LEC powerfully triggers effector and antigen-presenting functions of macrophages and enhances T cell cytotoxicity. J Leukoc Biol 75: 135-142.
10. Cappello P, Fraone T, Barberis L, Costa C, Hirsch E, et al. (2006) CC-chemokine ligand 16 induces a novel maturation program in human immature monocyte-derived dendritic cells. J Immunol 177: 6143-6151.
11. Caorsi C, Cappello P, Ceruti P, Amici A, Marchini C, et al. (2008) CCL16 enhances the CD8+ and CD4+ T cell reactivity to human HER-2 elicited by dendritic cells loaded with rat ortholog HER-2. Int J Immunopathol Pharmacol 21: 867-877.
12. Khawli LA, Hu P, Epstein AL (2008) Cytokine, chemokine, and co-stimulatory fusion proteins for the immunotherapy of solid tumors. Handb Exp Pharmacol : 291-328.
13. Chen S, Yu L, Jiang C, Zhao Y, Sun D, et al. (2005) Pivotal study of iodine-131-labeled chimeric tumor necrosis treatment radioimmunotherapy in patients with advanced lung cancer. J Clin Oncol 23: 1538-1547.
14. Flanagan ML, Khawli LA, Hu P, Epstein AL (2006) H60/TNT-3 fusion protein activates NK cells in vitro and improves immunotherapeutic outcome in murine syngeneic tumor models. J Immunother 29: 274-283.
15. Hdeib A, Sloan A (2012) Targeted radioimmunotherapy: the role of ^{131}I-chTNT-1/B mAb (Cotara) for treatment of high-grade gliomas. Future Oncol 8: 659-669.
16. Hornick JL, Sharifi J, Khawli LA, Hu P, Bai WG, et al. (2000) Single amino acid substitution in the Fc region of chimeric TNT-3 antibody accelerates clearance and improves immunoscintigraphy of solid tumors. J Nucl Med 41: 355-362.
17. Hornick JL, Sharifi J, Khawli LA, Hu P, Biela BH, et al. (1998) A new chemically modified chimeric TNT-3 monoclonal antibody directed against DNA for the radioimmunotherapy of solid tumors. Cancer Biother Radiopharm 13: 255-268.
18. Jang JK, Khawli LA, Park R, Wu BW, Li Z, et al. (2013) Cytoreductive chemotherapy improves the biodistribution of antibodies directed against tumor necrosis in murine solid tumor models. Mol Cancer Ther 12: 2827-2836.
19. Khawli LA, Biela B, Hu P, Epstein AL (2003) Comparison of recombinant derivatives of chimeric TNT-3 antibody for the radioimaging of solid tumors. Hybrid Hybridomics 22: 1-9.
20. Yu L, Ju DW, Chen W, Li T, Xu Z, et al. (2006) 131I-chTNT radioimmunotherapy of 43 patients with advanced lung cancer. Cancer Biother Radiopharm 21: 5-14.
21. Biela BH, Khawli LA, Hu P, Epstein AL (2003) Chimeric TNT-3/human beta-glucuronidase fusion proteins for antibody-directed enzyme prodrug therapy (ADEPT). Cancer Biother Radiopharm 18: 339-353.
22. Hornick JL, Khawli LA, Hu P, Sharifi J, Khanna C, et al. (1999) Pretreatment with a monoclonal antibody/interleukin-2 fusion protein directed against DNA enhances the delivery of therapeutic molecules to solid tumors. Clin Cancer Res 5: 51-60.
23. Mizokami MM, Hu P, Khawli LA, Li J, Epstein AL (2003) Chimeric TNT-3

antibody/murine interferon-gamma fusion protein for the immunotherapy of solid malignancies. Hybrid Hybridomics 22: 197-207.

24. Mitchell L, Thamm DH, Biller BJ (2012) Clinical and immunomodulatory effects of toceranib combined with low-dose cyclophosphamide in dogs with cancer. J Vet Intern Med 26: 355-362.

25. Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, et al. (2009) The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. Cancer Res 69: 2514-2522.

26. Pryer NK, Lee LB, Zadovaskaya R, Yu X, Sukbuntherng J, et al. (2003) Proof of target for SU11654: inhibition of KIT phosphorylation in canine mast cell tumors. Clin Cancer Res 9: 5729-5734.

27. Chon E, McCartan L, Kubicek LN, Vail DM (2012) Safety evaluation of combination toceranib phosphate (Palladia®) and piroxicam in tumour-bearing dogs (excluding mast cell tumours): a phase I dose-finding study. Vet Comp Oncol 10: 184-193.

28. London C, Mathie T, Stingle N, Clifford C, Haney S, et al. (2012) Preliminary evidence for biologic activity of toceranib phosphate (Palladia®) in solid tumours. Vet Comp Oncol 10: 194-205.

29. London CA, Hannah AL, Zadovoskaya R, Chien MB, Kollias-Baker C, et al. (2003) Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. Clin Cancer Res 9: 2755-2768.

30. Robat C, London C, Bunting L, Mc Cartan L, Stingle N, et al. (2012) Safety evaluation of combination vinblastine and toceranib phosphate Palladia® in dogs: a phase I dose-finding study. Vet Comp Oncol 10: 174-183.

31. Bernabe LF, Portela R, Nguyen S, Kisseberth WC, Pennell M, et al. (2013) Evaluation of the adverse event profile and pharmacodynamics of toceranib phosphate administered to dogs with solid tumors at doses below the maximum tolerated dose. BMC Vet Res 9: 190.

32. (2011) Veterinary cooperative oncology group - common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. Vet Comp Oncol.

33. Lechner MG, Russell SM, Bass RS, Epstein AL (2011) Chemokines, costimulatory molecules and fusion proteins for the immunotherapy of solid tumors. Immunotherapy 3: 1317-1340.

34. Ko JS, Rayman P, Ireland J, Swaidani S, Li G, et al. (2010) Direct and differential suppression of myeloid-derived suppressor cell subsets by sunitinib is compartmentally constrained. Cancer Res 70: 3526-3536.

35. Finke JH, Rini B, Ireland J, Rayman P, Richmond A, et al. (2008) Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. Clin Cancer Res 14: 6674-6682.

36. Goulart MR, Pluhar GE, Ohlfest JR (2012) Identification of myeloid derived suppressor cells in dogs with naturally occurring cancer. PLoS One 7: e33274.

37. Sherger M, Kisseberth W, London C, Olivo-Marston S, Papenfuss TL (2012) Identification of myeloid derived suppressor cells in the peripheral blood of tumor bearing dogs. BMC Vet Res 8: 209.

38. Pan X, Tsimbas K, Kurzman ID, Vail DM (2014) Safety evaluation of combination CCNU and continuous toceranib phosphate (Palladia®) in tumour-bearing dogs: a phase I dose-finding study. Vet Comp Oncol.