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Safety evaluation of combination doxorubicin and toceranib phosphate (Palladia®) in tumour bearing dogs: a phase I dose-finding study

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Abstract

Combination chemotherapy holds promise for improving outcomes in malignancy when compared with single-agent approaches. Care must be taken to avoid overlapping toxicity and to utilize agents with differing mechanisms of action. A phase I dose-finding trial was performed to determine the maximally tolerated dose (MTD) of a concurrent toceranib and doxorubicin (DOX) combination protocol where toceranib dose was maintained at or near 2.75 mg kg^{-1} by mouth every other day (PO EOD) while escalating DOX dosage. The dose-limiting toxicity was found to be neutropenia and the MTD of the combination was determined to be 25 mg m^{-2} of DOX q 21 days given concurrently with toceranib 2.75 mg kg^{-1} PO EOD. This combination was well tolerated with no excessive gastrointestinal toxicity nor novel adverse events (AEs) noted. Anti-tumour activity was observed in the majority of cases. This combination warrants further investigation in the context of phase II/III clinical trials to characterize efficacy and long-term AE profiles.

Keywords
cancer, canine, chemotherapy, doxorubicin, toceranib, Tregs

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Introduction

A seminal discovery in the evolution of cancer treatment was the advantage of combination chemotherapy regimens over single-agent therapy. There now exists ample clinical precedent for the use of multi-agent protocols, and almost all curative-intent protocols in human oncology incorporate a minimum of two or three chemotherapeutic agents with additive or synergistic activity. Ideally, such combination chemotherapy protocols adhere to several principles, in order to optimize clinical benefit.[1] The individual drugs should have proven single-agent activity against the tumour histotype being treated. The antitumour mechanism for each of the drugs should differ and, related to this, drugs with considerably similar adverse event (AE) profiles should be avoided. Finally, the drugs should be able to be administered at their optimal therapeutic dose and interval. Mindful of these criteria, our group has previously evaluated the combinations of toceranib phosphate (Palladia®) with piroxicam, vinblastine, and CCNU (lomustine) in dogs.[2–5] Further investigations of multi-drug chemotherapy protocols are warranted in veterinary oncology to strive for increased efficacy and improved outcomes in our

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companion animals with spontaneously arising tumours.

Doxorubicin (DOX) is an anthracycline derived from Streptomyces yeast species.[6] It has multiple mechanisms of cellular toxicity, including intercalation of DNA leading to inhibition of protein synthesis, free radical formation and topoisomerase enzyme inhibition. DOX is one of the most commonly utilized chemotherapy agents in veterinary medicine and has showed activity against a variety of histologies.[6,7] This broad spectrum of activity makes DOX an attractive agent to combine with other agents to further enhance antitumour activity. The AE profile of single-agent DOX in dogs is well established with myelosuppression, in particular neutropenia, being the acute dose-limiting AE and myocardial toxicity with cumulative doses over 180–240 mg m^{-2} being the chronic dose-limiting AE.[6,8] Other AEs common following DOX include alopecia and gastrointestinal AEs such as vomiting and diarrhoea, with the latter occasionally being dose-limiting.

Toceranib phosphate (Palladia; Zoetis, Florham Park, NJ) is a receptor tyrosine kinase inhibitor (RTKI) possessing inhibitory activity against several members of the receptor tyrosine kinase family of transmembrane proteins, including the cell surface receptors for vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), stem cell factor (SCF), cytokine Flt-3 ligand, and the GDNF-family ligands.[9,10] Toceranib's activity results from competitive blockade of the ATP-binding site of these tyrosine kinase receptors, resulting in impaired phosphorylation and diminished down-stream signalling.[11] In a phase I dose-finding study of toceranib, clinical responses were observed in a variety of tumour types including metastatic carcinomas, sarcomas, multiple myeloma and melanoma.[10] Subsequent off-label investigation further supported a role for toceranib in the treatment of numerous canine neoplasms, including osteosarcoma, thyroid carcinoma, apocrine gland anal sac adenocarcinoma, nasal carcinoma and head and neck squamous cell carcinomas.[12] Toceranib's most common AEs are gastrointestinal, especially diarrhoea.[10,13] Proteinuria and hypertension are also commonly seen secondary to toceranib use in our institution, and are also seen in humans secondary to RTKI use. The mechanisms of these toxicities have not been determined but are suggested to occur secondary to inhibition of VEGF.[14] Less frequent toxicities that have been reported in dogs include musculoskeletal pain, elevated liver enzymes and azotemia. Haematopoietic toxicity is uncommonly reported with toceranib and is typically mild if it is noted at all. Cardiotoxicity including left ventricular dysfunction, congestive heart failure and thromboembolic disease, is also uncommonly reported secondary to RTKI use in human patients.[14] To the authors' knowledge, these effects have not been commonly reported in canine patients. The complexity of the RTKIs' cellular interactions is not yet completely appreciated and there is ongoing research interrogating the potential roles of the receptor RTKIs as chemosensitizers, radiation sensitizers, immune modulators and in reversing acquired chemotherapy resistance. Toceranib has also been shown in one study to result in selective depletion of circulating regulatory T lymphocytes (Tregs) in tumour-bearing dogs when used as a single agent and when combined with metronomic cyclophosphamide.[15]

The hypothesis under investigation in this trial was that toceranib could be safely administered to dogs with naturally occurring malignancies on an every other day (EOD) schedule in combination with DOX administered every 3 weeks, at dosages with respective demonstrable single-agent activity. This study had three objectives. The primary objective was to employ a conventional 3 + 3 phase I study design to determine the maximum tolerated dose (MTD) of DOX that can be safely administered to tumour-bearing dogs with concurrent toceranib administered according to a standard dose and schedule.[16] The second objective was to characterize the AE profile of combination DOX and toceranib in dogs. A third objective was to characterize the effect of the DOX/toceranib combination protocol on circulating Tregs owing to toceranib's previously documented immunomodulatory single-agent effect on this lymphocyte subpopulation. Whilst efficacy is never a primary endpoint of such dose-finding studies, clinical benefit was also evaluated throughout the study period.

Methods

Patient selection

Client-owned dogs with one or more cytologically and/or histologically diagnosed malignant tumours of any histology (excluding mast cell tumours), grade and stage were considered for enrollment. Dogs with mast cell tumours were excluded given the investigators' concern regarding the paraneoplastic gastrointestinal effects observed with this histology, which could confound interpretation of gastrointestinal AEs secondary to proposed therapy, especially in this smaller study population. Prior surgery was allowed, hence, measurable macroscopic disease was not an inclusion criteria. Prior radiation therapy, chemotherapy (other than DOX or toceranib) and corticosteroid therapy were also allowed, with adherence to the predetermined minimum washout periods of 3 weeks for both radiation and chemotherapy, and 72 h for corticosteroid therapy. Concurrent non-steroidal anti-inflammatory therapy was permitted if it was instituted at least 30 days prior to enrollment. A complete physical examination, haematologic analysis [complete blood count (CBC)], serum biochemistry profile, urinalysis and blood pressure measurement were performed prior to initial treatment. Additional inclusion criteria included a minimum body weight of 10 kg, haematologic and biochemical parameters deemed adequate for the safe administration of chemotherapy, the absence of severe or uncontrolled concurrent disease(s), and a Veterinary Cooperative Oncology Group (VCOG) common terminology criteria for AEs (VCOG-CTCAE) v1.1 performance status of 0 (fully active, able to perform at pre-disease level) or 1 (activity less than pre-disease level, but able to function as an acceptable pet).[17] The clinical trial protocol was approved by the Animal Care and Use Committee of the School of Veterinary Medicine, University of Wisconsin-Madison, and written informed consent was obtained from owners prior to clinical trial commencement.

Study design and treatment protocol

A conventional open-label phase I dose-cohort 3 + 3 escalation study design was employed[16] with the DOX dose increasing with each cohort and the toceranib dose remaining constant. The initial cohort received DOX at a dose of 20 mg m^{-2} administered intravenously (IV) every 21 days for a total of four doses. DOX was escalated by 5 mg m^{-2} for each cohort until MTD or the standard dose of 30 mg m^{-2} was reached, whichever came first. The toceranib dose was maintained at approximately 2.75 mg kg^{-1} by mouth (PO) EOD throughout the trial period and extending for a minimum of 7 days following the fourth DOX dose. Toceranib dosing was necessarily approximate to accommodate the drug's commercially available tablet sizes. Owners were given the option of having their dog receive a fifth dose of DOX and/or continuing toceranib after trial conclusion.

Three dogs were enrolled in each cohort and then observed for dose-limiting toxicities (DLTs). A DLT was defined as any grade 3 or higher AE (VCOG-CTCAE v1.1[17]) with the exception of haematopoietic AEs (i.e. neutropenia, thrombocytopenia) for which a grade 4 AE was defined as dose-limiting. Furthermore, any grade AE that was refractory to supportive care or persisting beyond 7 days was also considered dose-limiting. If no AEs were observed within the first cohort of dogs following 3 weeks of treatment, a second cohort was treated with the DOX dose escalated by 5 mg m^{-2}. If a DLT was observed in one of the three dogs, the cohort was expanded to a total of six dogs. If two or more DLTs were noted in any cohort, it was considered that the MTD had been exceeded. Therefore, the MTD was defined as the highest dose level at which no more than one of six dogs developed a DLT.

Assessment of AEs

All dogs were evaluated at the University of Wisconsin Veterinary Medical Teaching Hospital (UW-VMTH) prior to trial enrollment. Physical examination, haematologic analysis (CBC), serum biochemistry profile, urinalysis and blood pressure measurement were performed at baseline, then every 21 days immediately prior to each DOX dose. The urine protein:creatinine ratio (UPC) was calculated if the urinalysis and/or blood pressure measurement were suggestive of pathologic

proteinuria and/or hypertension. No specific cardiac monitoring was performed unless dictated by changes (such as new murmur, arrhythmia) noted on physical examination. As our protocol would result in cumulative doses of less than 180 mg m^{-2}, and previous evidence has demonstrated that routine echocardiogram or electrocardiogram (ECG) monitoring is insensitive for detection of DOX-induced cardiotoxicity in non-symptomatic patients, this was not deemed necessary for patient assessment.[18] Physical examination and CBC were also performed by clinicians at the UW-VMTH or the referring veterinarian 1 week following each DOX dose.

All AEs were characterized and graded according to VCOG-CTCAE v1.1.[17] Disease progression or clinical signs that were attributed to the underlying disease were not considered AEs. Permissible supportive care for gastrointestinal AEs included a 7-day toceranib drug holiday, anti-diarrhoeal medications, such as metronidazole and tylosin, antiemetics, such as maropitant, ondansetron and metoclopramide, and gastric protectants, such as famotidine, ranitidine and omeprazole. Hypertension was managed with angiotensin converting enzyme (ACE) inhibitors, either benazepril or enalapril, and/or amlodipine as recommended by the UW-VMTH cardiology service.

Antitumour response assessment

Tumour response was assessed for those dogs with macroscopically measurable disease on study days 0, 21, 42, 63 and 84. Follow-up after day 84 varied because not all patients continued therapy or monitoring. Tumour response was assessed according to either VCOG response evaluation criteria for solid tumours (RECIST) in dogs v1.0[19] or VCOG v1.0[20] response evaluation criteria for peripheral nodal lymphoma as was case applicable. Tumour measurement was performed as indicated using callipers, thoracic radiographs, ultrasonography and/or computerized tomography (CT) with the associated imaging programme. A complete response (CR) was defined as complete regression of all measurable disease; partial response (PR) was defined as at least a 30% reduction in the sum of the target lesion(s') longest diameter(s); progressive disease (PD) was defined as at least a 20% increase in the sum of the target lesion(s') longest diameter(s) or the appearance of new lesions; stable disease (SD) was defined as neither CR, PR nor PD for at least 6 weeks. Dogs that developed PD were withdrawn from the trial and owners were offered alternative treatment options. Progression-free survival (PFS) was defined as the time (days) from the start of treatment to documented progression (local and/or distant) or death from any cause, for cases with macroscopic disease, or time to documented recurrence, metastasis or death from any cause for cases with microscopic disease at treatment initiation. Patients were censored if still alive at last assessment prior to manuscript preparation.

Peripheral blood mononuclear cell (PBMC) sample preparation

In all dogs in the 25 mg m^{-2} and higher DOX cohorts, an additional 15 mL of ethylenediaminetetraacetic acid (EDTA) whole blood was collected immediately prior to the initial DOX dose and then every 21 days, immediately prior to each subsequent DOX dose, for peripheral blood mononuclear cell (PBMC) enumeration and lymphocyte subset flow-cytometric analysis. PBMCs were isolated from the EDTA blood using density gradient separation. Briefly, whole blood was diluted 1:1 in Hanks' balanced salt solution (HBSS) followed by 10 mL of lymphocyte separation medium (Cellgro, Manassas, VA, USA). Following centrifugation at $830 \times g \times 18$ min, PBMCs were removed from the gradient and washed in HBSS. Cells were then centrifuged at 340 $g \times \times 7$ min and red blood cells were lysed by adding ACK red cell lysis buffer (Lonza, Walkersville, MD, USA). Cells were then washed in HBSS, centrifuged and re-suspended in lymphocyte freezing medium [90% foetal bovine serum (FBS)/10% demethyl sulfoxide (DMSO)] at a concentration of $\sim 5.0 \times 10^6$ cells mL^{-1}. Vials containing the cells were placed at −80° C and frozen at a rate of cooling of -1^{o} C min^{-1} in a Mr. Frosty freezing container (Thermo Scientific, Waltham, MA, USA). After 24 h, cells were transferred to liquid nitrogen and stored until shipment. Samples were shipped in batch on dry ice to the laboratory

of one author (B. J. B.) for lymphocyte subset analysis.

Flow cytometric lymphocyte subset analysis

Cryopreserved PBMC samples were batch analysed for Treg subset analysis in the laboratory of Dr. Barb Biller at Colorado State University using previously published and validated techniques.[15,21–23] Briefly, samples were thawed and the PBMCs washed, then added to a 96-well plate at a concentration of 5×10^5 per well. All samples were treated for 5 min with a mixture of normal canine serum, human IgG and anti-mouse CD16/32 antibodies to block non-specific binding. Samples were then immunostained for surface expression of CD4, CD8 and CD25 with the following reagents respectively: Pacific Blue conjugated anti-canine CD4 (clone YKIX302.9, Serotec [Raleigh, NC, USA], Raleigh, NC, USA), Alexa-647 conjugated anti-canine CD8, clone YCATE 55.9, Serotec), and FITC conjugated anti-dog CD25 (clone P4A10, eBioscience, San Diego, CA, USA). Following surface staining, the cells were washed then fixed and permeabilized using the Foxp3/Transcription factor staining buffer set (eBioscience) according to the manufacturer's instructions. Samples were then stained intracellularly with PE-conjugated anti-mouse/rat Foxp3 (clone FJK-16s, eBioscience). Flow cytometry was performed using a Cyan ADP flow cytometer (Beckman Coulter, Miami, FL, USA) and the data analysed using FlowJo analysis software (FlowJo, Ashland, OR, USA). Live lymphocytes were gated based on forward and side-scatter properties, then analysed for CD4 and CD8 expression. The percentage of Tregs was determined based on percentage of $CD4^+$ T cells that expressed both Foxp3 and CD25. The percentages of $CD4^+$ and $CD8^+$ T cells were also determined. Absolute numbers of Tregs in peripheral blood were then calculated based on the total lymphocyte count determined from a CBC obtained from the same blood draw as part of the clinical monitoring.

Statistical analyses

Changes in total lymphocytes and Tregs over time from dogs receiving the combination protocol at or above DOX MTD were compared using a non-parametric repeated measures analysis of variance (ANOVA) analysis (Friedman's) and Dunn's Multiple Comparison *post hoc* Test for comparison of individual time points. All statistical analyses were performed with a commercial software package (Prism v5.0, GraphPad Software, LaJolla, CA, USA). *P* values less than 0.05 were considered statistically significant.

Results

Patient demographics

A total of 21 dogs were enrolled between January 2014 and February 2015. Two dogs were not evaluable because they received only a single DOX dose with no follow-up blood work as a result of disease progression and euthanasia ($n = 1$) or owner withdrawal ($n = 1$); these patients never received toceranib. Three additional dogs were considered only minimally evaluable because they received only the initial DOX dose and very short-term toceranib before being withdrawn from the study because of disease progression. Whilst these three dogs did have a CBC performed 7-days post-DOX with no dose-limiting AEs, no further monitoring was performed. A total of 16 dogs were, therefore, evaluable. Their demographic characteristics are presented in Table 1. Of these, 12 dogs had the diagnosis of neoplasia made via histopathology, whereas four had the diagnosis made via cytology. Four dogs had received previous chemotherapy.

Dose and safety evaluation

The number of dogs treated in each cohort and number of DLTs per cohort are presented in Table 2. AEs per cohort and grade are presented in Table 3. No deaths occurred as the result of combination therapy. The mean and median dosages of toceranib received were 2.67 mg kg^{-1} PO EOD and 2.7 mg kg^{-1} PO EOD, respectively (range: 2.25–2.81 mg kg^{-1} PO EOD). A total of 14 patients (87.5%) received the intended four DOX doses. One dog received only one dose and another dog received three doses before disease progression was noted and trial participation ceased. The patient receiving only one DOX dose did receive 21 days

Table 1. Patient demographics

Patient demographics and outcome			
Age (years)	Median	9.2	
	Range	6.7–14.8	
Weight (kg)	Median	28.2	
	Range	13.3-53.5	
Sex	MN	5	
	MI	2	
	FS	9	
Breed	Labrador retriever	5	
	Pit bull terrier	3	
	Golden retriever	2	
	Beagle, Brittany, GSD, Samoyed, Alaskan Malamute, mixed breed	1 each	
Tumour types	Hemangiosarcoma	8	PFS (days)
	Splenic stage I	1	197
	Splenic stage II	2	112, 225
	Splenic stage III	2	122, 176
	Right auricular	1	146
	Intramuscular	1	199
	Retroperitoneal	1	92
	Multicentric lymphoma	3	189, 159, 64
	Anal sac adenocarcinoma	2	228, 232
	Prostatic carcinoma[a]	2	281, 135
	Cutaneous lymphoma	1	22

GSD, German Shepherd Dog; MN, male neutered; MI, male intact; FD, female spayed.
[a]This patient also had a splenic sarcoma.

of toceranib and did have appropriate monitoring at day 21 for AEs. The dose-limiting toxicity of combination DOX and toceranib was determined to be myelosuppression, specifically neutropenia. In the 30 mg m^{-2} DOX cohort, two out of five dogs developed a febrile grade 4 neutropenia 1 week after the first or second DOX dose, and one dog developed a febrile grade 3 neutropenia 2 weeks after receiving DOX. One of the patients in the 30 mg m^{-2} DOX cohort who developed febrile neutropenia did not have further neutropenic episodes with a 20% dose reduction and went on to receive two additional DOX doses (a total of four doses). The other patient continued to have grade 4 febrile neutropenia episodes despite 20% dose reductions; it should also be noted that this patient had other febrile neutropenia episodes after other chemotherapy agents that were received prior to being enrolled in the clinical trial. Within other cohorts, neutropenia without fever (grade 3) and thrombocytopenia (grade 3) were also seen. One dog who received DOX at a dose of 25 mg m^{-2} developed a grade 4 febrile neutropenia 2 weeks following the fourth DOX dose. Previous DOX doses had been well tolerated and a CBC 8 days after the fourth DOX dose was within normal limits. This patient was concurrently diagnosed with a urinary tract infection and it is unknown if this contributed to the febrile neutropenic episode or was a consequence of the neutropenia. This patient responded well to supportive care of hospitalization with intravenous fluids and antibiotics. This was the only patient in the 25 mg m^{-2} cohort experiencing DLT, and as a result, the MTD of DOX, when given concurrently with toceranib at ~2.75 mg kg^{-1} PO EOD, was established as 25 mg m^{-2} IV every 21 days. Owing to availability of funds and drugs, two additional dogs were subsequently treated at the 25 mg m^{-2} cohort bringing the total number treated in this cohort to 8.

Gastrointestinal AEs (vomiting, diarrhoea, colitis, hyporexia or anorexia) were also observed with the combination of DOX and toceranib. Most of these AEs were mild and self-limiting and responded to supportive care. A total of three dogs received a 7-day toceranib drug holiday for treatment of gastrointestinal side effects (colitis, diarrhoea and anorexia). All three dogs had

Table 2. Scheduled treatment cohorts

Cohort	Toceranib dose (mg kg^{-1} EOD)	DOX (mg m^{-2}, q21 days)	Dogs treated	Number of dogs experiencing DLT
1	2.75	20	3	0
2	2.75	25	8	1
3	2.75	30	5	3

Table 3. AEs by grade and cohort

		Cohort		
AE classification	**Grade**	**1 ($n = 3$)**	**2 ($n = 8$)**	**3 ($n = 5$)**
Neutropenia	1	1	1	1
	2	1	3	
	3		1	2
	4		1	2
Thrombocytopenia	1	1		2
	2	1	3	1
	3			2
Anaemia	1		6	2
	2		1	1
Anorexia	1	1	2	
	2			2
Weight loss	1		1	
	2		1	
Lethargy	1			
	2		1	
Vomiting	1			1
Diarrhoea	1	1	1	2
	2		3	
Colitis	2		3	
Elevated ALT	1	1	3	1
	2	1	1	
Elevated AST	1	1	5	2
Elevated ALP	1		2	3
	2	1		
Hypertension	1	1	1	
	2		2	2
	3		1[a]	
Elevated BUN	2		1	
Elevated creatinine	1	1	1	1
Proteinuria	1		1	
	2		1	1
Arrhythmia	2		1	
Left ventricular diastolic dysfunction	2		1	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen.
[a]Patient was hypertensive at baseline and managed with amlodipine and enalapril.

resolution of their clinical signs during the drug holiday and toceranib therapy was reinstituted without recrudescence of the clinical signs. One dog did have toceranib discontinued after 2 weeks because of persistent anorexia. This dog developed PD and only received one DOX dose. Another dog who experienced anorexia was concurrently receiving enalapril and amlodipine for proteinuria and hypertension, and this dog's appetite improved when these drugs were discontinued.

Proteinuria was pre-existing in two dogs at enrollment. Proteinuria remained stable in one dog for the duration of therapy. The other dog had progression of proteinuria; it is unknown whether this was due to therapy or progression of underlying disease. Two dogs developed proteinuria during the trial. An ACE inhibitor was commenced in both cases (enalapril 0.5 mg kg^{-1}), and proteinuria improved on subsequent evaluations.

Hypertension was noted in seven patients, although four of these patients were hypertensive at baseline. Two patients who developed hypertension during the clinical trial had concurrent disease that can also be associated with hypertension (diabetes mellitus, pre-existing proteinuria) and therefore it is difficult to determine the true contribution of toceranib to these AEs. All patients were able to have their hypertension controlled with oral medical therapy (ACE inhibitors, amlodipine); two patients did receive both medications for dual control of proteinuria and hypertension. One additional patient received both medications for control of hypertension but this patient was hypertensive at baseline prior to receiving DOX or toceranib.

Elevated liver enzymes (ALT, ALP and AST) and renal values (creatinine, BUN) were noted in a total of 25 patients. These elevations were of low grade and transient. Grade 1 aspartate aminotransferase (AST) elevation was most common amongst these elevations and occasionally occurred without elevations of other enzymes. These elevations have been previously reported with toceranib and may be related to the idiopathic musculoskeletal pain

and lameness that has been noted with use of the drug.[13,24] No dogs developed musculoskeletal pain or lameness, or evidence of liver dysfunction during this clinical trial. Two dogs which developed elevated liver enzymes were concomitantly diagnosed with progressive lymphoma and this could also have contributed to the elevations. One of these dogs was also concurrently receiving prednisone therapy at the time of day 21 blood work due to poor appetite, despite this being a contraindication for the clinical trial. It is probable that prednisone also contributed to elevated ALT and ALP.

One patient in the 25 mg m^{-2} cohort was diagnosed with supraventricular tachycardia on ECG after an elevated heart rate was detected on physical examination prior to the third DOX dose. This patient was treated with diltiazem which converted the arrhythmia to normal sinus rhythm. This patient went on to have two subsequent DOX doses. This patient had progression of disease (initially stage I splenic hemangiosarcoma) noted after 197 days and was subsequently treated with multiple other chemotherapy agents (cyclophosphamide, vincristine, carboplatin). Six months after receiving the last DOX the patient was noted to have a new arrhythmia and an echocardiogram was performed showing mild left ventricular systolic dysfunction. This was treated with mexiletine and pimobendan. This was believed to be secondary to DOX; no right atrial mass was seen. The patient was ultimately euthanized due to PD.

The patient in the 30 mg m^{-2} cohort who experienced multiple episodes of febrile neutropenia despite DOX dose reductions was diagnosed with severe left ventricular systolic dysfunction and mild congestive heart failure after presenting for cough and syncopal episodes 2 months after the fourth DOX dose. This was believed to be secondary to DOX. The patient was successfully treated for heart failure and died due to complications from diabetes mellitus.

Response and outcome assessment

Eight dogs had macroscopic measurable disease at the time of enrollment. Of these, one CR (multicentric lymphoma, PFS = 189 days), three PR (two multicentric lymphoma, PFS = 159 and 64 days; one intramuscular hemangiosarcoma, PFS = 199 days), three SD (two prostatic carcinoma, PFS = 281 and 135 days; 1 stage III splenic hemangiosarcoma, PFS = 122 days) and one PD (cutaneous lymphoma) were observed.

Eight dogs had surgical removal of all measurable disease and commenced the trial in the microscopic disease setting: four splenic hemangiosarcoma (PFS = 112, 176, 197, 225 days), two anal sac adenocarcinoma (PFS = 228 and 232 days) and one each of right auricular hemangiosarcoma (PFS = 146 days) and retroperitoneal hemangiosarcoma (PFS = 92 days). The two patients with anal sac adenocarcinoma were still alive and censored at time of last evaluation. The previously noted patient died secondary to complications of diabetes mellitus with stable disease. All other patients had PD or died due to their disease.

Effect of simultaneous combination treatment on peripherally circulating lymphocytes and Tregs

In dogs receiving the combination of toceranib ~2.75 mg kg^{-1} PO EOD and DOX 25 or 30 mg m^{-2} IV q 21 days, no statistically significant declines were observed in circulating total lymphocytes or in absolute or relative (percent) Treg lymphocyte numbers, when measured at 21 day intervals (Figure 1). PBMCs and therefore Tregs were not collected in patients receiving the lowest DOX cohort 20 mg m^{-2}.

Discussion

The MTD for the combination protocol was determined to be DOX administered intravenously at 25 mg m^{-2} every 21 days and toceranib administered PO at 2.75 mg kg^{-1} EOD. The DLT of the combination of DOX and toceranib was myelosuppression, specifically neutropenia. No AEs were noted with this combination that would not have been predicted based upon the known AE profiles for either drug. It should be noted that some dogs did tolerate the higher (30 mg m^{-2}) DOX dose in combination with toceranib and therefore, as with what should be standard oncology practice, DOX dose increases should be considered in those patients tolerating 25 mg m^{-1} when a lack of appropriate nadir

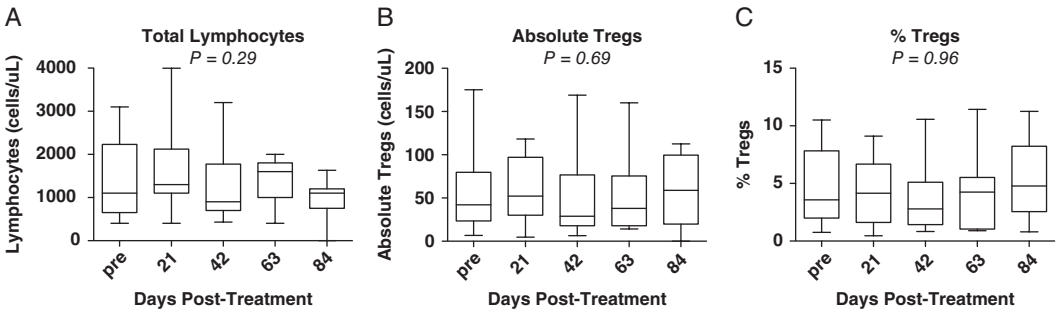

Figure 1. Box and traditional Tukey whiskers plots of total lymphocytes and Treg numbers and proportion following simultaneous combination of DOX and toceranib in tumour-bearing dogs. The medians, 75 percentile (box) and whiskers (1.5 times the interquartile distance or the highest or lowest point, whichever is shorter) are depicted. (A) Total circulating lymphocytes did not change significantly over time after treatment ($P = 0.29$). (B) Absolute Tregs did not change significantly over time after treatment ($P = 0.69$). (C) Relative (percent) Tregs did not change significantly over time after treatment ($P = 0.96$).

is observed in order to ensure maximum potential efficacy.

The DLTs of neutropenia observed in this study in dogs in the 30 mg m^{-2} DOX cohort along with 2.75 mg kg^{-1} toceranib was more marked than is known to occur with either agent alone at those dosages. Previous trials performed by our group and others have also showed apparent sensitization of the myeloid compartment by toceranib as evidenced by enhanced myelosuppression noted when toceranib is used in combination with CCNU and vinblastine.[2–4] In human clinical trials, similar findings have been observed with the combination of cytotoxic agents and RTKIs including gefitinib/gemcitabine, gefitinib/vinorelbine, erlotinib/vinorelbine and vinca alkaloids/EGFR inhibitor combination protocols.[25–28] One suggested mechanism for this increased haematologic toxicity is that the RTKIs, DOX and vinca alkaloids are all metabolized by CYP3A4 enzymes, resulting in prolonged systemic exposure to the active metabolites due to saturation of hepatic metabolism when these agents are combined.[29,30] Another potential explanation is that toceranib and similar drugs inhibit colony stimulating factor-1 receptor, which could exacerbate the level of neutropenia seen when combined with cytotoxic drugs.[24] In addition, several RTKIs have been shown to inhibit ATP-binding cassette (ABC) multidrug resistant transporters (MDR-ABC proteins).[31] Sunitinib, a RTKI that targets identical RTKI receptors as toceranib, as well as imatinib mesylate and masitinib have specifically been shown to inhibit MDR systems.[32–36] As DOX is a known MDR substrate of both ABCB1 and ABCG2[37] this could, in part, explain the enhanced myelosuppression seen in combination with toceranib. Theoretically, this may also lead to decreased incidence of DOX resistance and increased tumour cytotoxicity when used in combination, although no supporting evidence for this theory is nor would be evident in a small phase I study such as this. While most episodes of neutropenia or thrombocytopenia occurred at the expected nadir for DOX (day 7), two patients did appear to have a more delayed nadir. This can occasionally be seen secondary to DOX administration, but it is possible that the toceranib administration could have affected this as well.

When used in a single agent setting both of these drugs can lead to gastrointestinal toxicity, therefore potentiation of these toxicities was a concern with combination. While gastrointestinal toxicities were seen in a number of patients, they were mild and transient and were successfully managed with symptomatic care and supportive medications.

Cardiotoxicity is an established cumulative toxicity of DOX and has been reported in human patients with RTKI use.[14] Possible DOX-related cardiotoxicity was seen in two patients in this study. Neither patient succumbed to cardiac disease. Post-mortem exam was not performed in either patient, so it is difficult to truly establish whether this was secondary to DOX or other

underlying disease. Given the short follow-up period inherent in phase I trials, it is not possible to adequately access whether the combination of DOX and toceranib alters the severity or incidence of DOX associated cumulative cardiac changes; such a characterization will await further evaluations in a phase II/III trial context.

While efficacy is not a primary endpoint of phase I clinical trials, antitumour activity was observed in the majority of cases treated in the macroscopic disease setting with this combination. Current dogma instructs that antitumour activity may be compromised if dose reductions are required to safely combine active agents into a combination protocol. This is of concern as in all trials investigating the concurrent combination of toceranib with standard cytotoxic agents (e.g. vinblastine, CCNU).[2–4] enhanced myelosuppression has been observed, resulting in an MTD for the cytotoxic agent that is approximately 20% less than that when the agent was used as a single-agent. However, this concern may be overstated owing to the response rates observed in these trials despite the dose reduction, and the inhibition of MDR systems that may result in enhanced activity when RTKIs are used in combination with MDR substrate chemotherapeutics. Further exploration of efficacy with these combinations in the context of phases II and III clinical trials will be necessary to determine if improved response rates and durations result. This study, as with any phase I trial with small patient numbers, a variety of tumour types, and short follow-up time, is not designed to show improved survival times.

No significant declines in absolute or relative (percent) Treg lymphocytes were observed in dogs undergoing combination therapy in this study. Previously, Mitchell reported statistically significant decreases in both absolute and relative (percent) Tregs in dogs receiving single-agent toceranib at similar doses to those utilized in this study when measured at 14 days post-initiation of toceranib therapy.[15] This decline was maintained for up to 56 days, but interpretation of subsequent measurements were complicated by the addition of metronomic cyclophosphamide beginning at day 14. Rasmussen et al.[38] reported that the addition of MTD-DOX treatment to metronomic cyclophosphamide, while resulting in decreased total lymphocyte and absolute Treg subpopulations, did not selectively decrease Treg numbers and therefore, did not result in a decrease in the percent of Treg populations as is reported to occur following metronomic cyclophosphamide.[21] Here, it was hypothesized that the addition of MTD-DOX resulted in indiscriminant lymphocyte depletion which overshadowed the selective Treg depletions observed when metronomic cyclophosphamide is used alone.[21] This finding, however, was in contrast to another report by Mitchell et al.[22] who found that in a small number of dogs with lymphoma, a single treatment of MTD–DOX resulted in a selective decrease in peripherally circulating Tregs at day 7 post-treatment. The lack of observable declines in total lymphocytes, as well as absolute and relative (percent) Treg numbers, in this study could be the result of some as of yet unknown interaction between the two agents used including the possibility that the Treg subset of lymphocytes is more sensitive to storage and processing. Any comparisons between this study and those of Mitchell and extrapolated conclusions are, however, inherently flawed because of the varied tumour types included, small population numbers, and the fact that in this study lymphocyte subpopulations were quantified 21 days after treatment initiation, rather than at 14 days as in the Mitchell study.[15]

The limitations of this study are similar to those inherent in all phase I dose-finding clinical trials. These include small patient numbers and short follow-up periods. Phases II and III clinical trials are needed to more accurately assess response rates and durability, as well as the combination protocol's long-term AE profile.

Conclusions

In general, the combination of DOX and toceranib was well tolerated. The MTD and schedule for combination was determined to be DOX 25 mg m^{-2} every 21 days and toceranib 2.75 mg kg^{-1} EOD. This concurrent combination shows an approximately 17% dose reduction for DOX when compared with its MTD as a single-agent. Despite this dose reduction, responses to the protocol were seen and enhanced myelosuppression was noted, indirectly suggesting activity. Prospective randomized phase

II/III trials are needed to further characterize activity and the chronic/cumulative AE profile of the combination relative to single-agent protocols.

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