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Authors

Barnard, Rebecca A
Wittenburg, Luke A
Amaravadi, Ravi K
et al.

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Phase I clinical trial and pharmacodynamic evaluation of combination hydroxychloroquine and doxorubicin treatment in pet dogs treated for spontaneously occurring lymphoma

Rebecca A Barnard,[1] Luke A Wittenburg,[1] Ravi K Amaravadi,[2] Daniel L Gustafson,[1] Andrew Thorburn,[3] and Douglas H Thamm[1,*]

[1]Department of Clinical Sciences; Colorado State University; Fort Collins, CO USA; [2]Department of Medicine Hematology Oncology Division; Pearlman School of Medicine; University of Pennsylvania; Philadelphia, PA USA; [3]Department of Pharmacology; University of Colorado School of Medicine; Aurora, CO USA

Autophagy is a lysosomal degradation process that may act as a mechanism of survival in a variety of cancers. While pharmacologic inhibition of autophagy with hydroxychloroquine (HCQ) is currently being explored in human clinical trials, it has never been evaluated in canine cancers. Non-Hodgkin lymphoma (NHL) is one of the most prevalent tumor types in dogs and has similar pathogenesis and response to treatment as human NHL. Clinical trials in canine patients are conducted in the same way as in human patients, thus, to determine a maximum dose of HCQ that can be combined with a standard chemotherapy, a Phase I, single arm, dose escalation trial was conducted in dogs with spontaneous NHL presenting as patients to an academic, tertiary-care veterinary teaching hospital. HCQ was administered daily by mouth throughout the trial, beginning 72 h prior to doxorubicin (DOX), which was given intravenously on a 21-d cycle. Peripheral blood mononuclear cells and biopsies were collected before and 3 d after HCQ treatment and assessed for autophagy inhibition and HCQ concentration. A total of 30 patients were enrolled in the trial. HCQ alone was well tolerated with only mild lethargy and gastrointestinal-related adverse events. The overall response rate (ORR) for dogs with lymphoma was 93.3%, with median progression-free interval (PFI) of 5 mo. Pharmacokinetic analysis revealed a 100-fold increase in HCQ in tumors compared with plasma. There was a trend that supported therapy-induced increase in LC3-II (the cleaved and lipidated form of microtubule-associated protein 1 light chain 3/LC3, which serves as a maker for autophagosomes) and SQSTM1/p62 (sequestosome 1) after treatment. The superior ORR and comparable PFI to single-agent DOX provide strong support for further evaluation via randomized, placebo-controlled trials in canine and human NHL.

Introduction

(Macro) autophagy is a lysosomal degradation process which allows for the recycling of cytosolic proteins and organelles.[1] Autophagy is therefore often upregulated in response to stress, acting as a quality control mechanism as well as replenishing amino acid, lipid, and nucleic acid pools.[2-5] These roles for autophagy are thought to enhance survival and therapy resistance in a variety of cancers.[6,7] A number of preclinical cancer therapy mouse xenograft models have been used to demonstrate the enhanced efficacy of cancer therapies when combined with the autophagy inhibitors chloroquine (CQ) or hydroxychloroquine (HCQ).[8-10] Thus, autophagy appears to be an attractive pathway to target. However, autophagy has been shown to have a protective role in a variety of organs including the gut, kidneys, and liver and combining HCQ with other drugs may exacerbate the toxicities of chemotherapy especially in these tissues.[11-13] Induction of autophagy may also enhance the cytotoxicity of certain drugs.[14-16] Therefore, establishing the safe and tolerable dose of HCQ in combination with standard of care drugs for canine cancers is an important first step in demonstrating the potential benefit of autophagy inhibition for these diseases.

Currently, the only autophagy inhibitor being used clinically is HCQ, a 4-aminoquinolone drug historically used in treatment of malaria and autoimmune diseases such as systemic lupus erythematosis and rheumatoid arthritis. HCQ pharmacokinetics are characterized by an extremely prolonged terminal half-life (up to 40 d) and a very large volume of distribution, in part due to the partitioning of the drug into red blood cells and strong binding to heme proteins.[17,18] In humans, HCQ is rapidly and almost completely absorbed following an oral dose with approximately 50% being bound to plasma proteins. Three HCQ metabolites have been identified, including N-desethylchloroquine, desethylhydroxychloroquine (DHCQ), and bidesethylchloroquine.[18,19] However, little is known about HCQ pharmacokinetics in dogs. HCQ has been used in canine discoid and cutaneous lupus erythematosus, similar to humans, at dosages of 5 to 10 mg/kg/d with some evidence of clinical efficacy, suggesting adequate blood and tissue concentrations for that specific indication.[20,21] An early laboratory study evaluating chloroquine and HCQ administration in dogs demonstrated that doses up to 32 mg/kg/d for 13 wk were well tolerated, with no significant alterations of liver function or hematology.[22] When compared with chloroquine administration, equivalent doses of HCQ were better tolerated and provided higher blood and tissue concentrations. However, the study did not evaluate pharmacokinetic parameters of HCQ in dogs following repeat administration. Additionally, the use of HCQ in treatment of canine cancer has yet to be studied.

The translational utility of the canine cancer model is based in the greater similarity to humans in terms of carcinogenesis and tumor biology. Dogs are relatively outbred, immunocompetent animals that share environments with humans, and experience spontaneously developing tumors with spontaneous metastasis and therapy resistance, representing a spectrum of tumor histotypes similar to humans. The relatively large size of canine tumors closely approximates human solid tumors in regard to biological factors such as clonal variation and hypoxia, and this relatively large size allows for multiple sampling of tumor tissues over time for pharmacodynamic assessments.[23,24] In addition, when compared with humans, disease progression in dogs is accelerated which allows for more rapid assessment of therapeutic endpoints than might be possible in similar human trials.[23,24] Pet dogs with spontaneous cancer have been utilized for the clinical evaluation of multiple novel cytotoxic, targeted, and immunomodulatory therapies. These studies have then been used to inform the design of further human clinical trials as well as determining treatment protocols in veterinary medicine.[25-32]

Therefore, canine clinical trials are designed like and have the same objectives (e.g., a Phase I trial is intended to determine doses and assess pharmacokinetics) as human clinical trials but with the possibility of more rapid and complete assessments than is often possible in humans.

Canine non-Hodgkin lymphoma (NHL) is a useful naturally occurring model of NHL in humans owing to significant similarities in pathogenesis, histology/biology, and response to treatment. Correlations between genetic factors and the development or progression of NHL have been identified in both canine and human NHL. In dogs, the main aneuploidies observed in NHL include gains of chromosomes 13 and 31, which are analogous to the partial gains of human chromosomes 4 and 8 and a gain of chromosome 21.[33] Subchromosomal regions of CFA 13/HSA8 and CFA31/HSA21 harbor genes important in tumorigenesis such as *c-myc*, frequently involved in human B-cell lymphomas through aberrant fusion with immunoglobin genes.[34] Canine and human peripheral T-cell lymphomas also demonstrate some conservation of copy number aberrations with both having deletions in chromosomal regions leading to loss of CDKN2A/B and CDKN2A/p16-RB1 pathway activity.[35,36] In addition to chromosomal aberrations, epigenetic changes such as methylation of CpG islands and promoter hypermethylation of orthologous tumor-suppressor genes have been identified in human and canine NHL.[37,38] In dogs, NHL is one of the most prevalent tumor types, making up 7–24% of all cancers and 83% of hematopoietic cancers.[39] The incidence of canine NHL (15–30/100,000) is very similar to that seen in human NHL (15.5–29.9/100,000).[23] Based on the REAL/WHO or National Cancer Institute Working Formulation schema, canine NHL represents a relatively homogenous population with respect to histologic type with 70% classified as medium to high grade B-cell NHL and the majority being diffuse large B-cell lymphoma.[40] At presentation, most dogs with NHL are asymptomatic and have generalized, nonpainful enlargement of peripheral lymph nodes. As in humans, canine NHL is initially highly responsive to multiagent, CHOP-based chemotherapy which is likely to yield complete responses in approximately 90% of dogs.[39] However, the duration of first remission is short and 85% of cases will relapse within 6 to 11 mo.[39] Owing to toxicity and cost of multiagent chemotherapy, single agent doxorubicin is a frequently used alternative that produces a substantially lower response rate of 63 to 85% and a median progression-free survival of approximately 5 mo.[41-43]

Although there have been reports of enhancement of antitumor activity with combinations of cytotoxic drugs and autophagy inhibition in vitro and with in vivo mouse models, there are no reports on the clinical utility of autophagy inhibition using HCQ in canine cancer patients. Here we report the results of a Phase I/II clinical trial of oral HCQ given continuously, starting 72 h prior to a standard dose of DOX. This trial was conducted in client-owned (pet) dogs with spontaneous neoplasia presenting as patients to an academic, tertiary-care veterinary teaching hospital. As would be the case in early-phase human clinical trials, primary endpoints included maximum tolerated dose, dose-limiting toxicities, and pharmacokinetic/pharmacodynamic relationships. Preliminary evidence of antitumor activity was also assessed.

Results

Dose-escalation trial

A 3 × 3 dose escalation trial design was used to govern dose escalation toward a maximum tolerated dose (MTD) of HCQ that could be tolerated when administered concurrently with

standard dosages of DOX (30 mg/m² given once every 3 wk) in dogs with any spontaneously occurring tumor. In all, 30 dogs met the inclusion criteria and were enrolled in the study beginning in February 2011 and running through September 2013. All dogs underwent pretreatment evaluation and blood chemistry and CBC, and pretreatment biopsies were obtained from accessible tumors. Patient parameters such as age, sex, weight, breed, and tumor type were recorded for each patient (**Table 1**). In all, 27 of 30 (90%) dogs presented with multicentric lymphoma; 24 (88.9%) and 3 (11.1%) were identified as B- and T-cell lymphoma, respectively. At enrollment, 2 of 30 dogs (6.7%) had documented pulmonary metastasis (1 fibrosarcoma and 1 osteosarcoma) and 2 of 30 had received prior chemotherapy. One dog with lymphoma had been previously enrolled in, and failed, a separate clinical trial.

Oral HCQ was well tolerated in dogs with no grade 3 or 4 toxicities attributable to the HCQ in any of the dose cohorts in the 3 d prior to DOX administration. Side effects of the HCQ were generally mild and self-limiting and mostly grade 1 or 2 lethargy and/or gastrointestinal upset (**Table 2**). A total of 112 treatment cycles were administered with an average of 3.7 per patient (range, 1–5). In all, 9 of 30 dogs (30%) required dose reductions in DOX following the first treatment cycle because of grade 3 or 4 toxicities attributable to DOX. The first HCQ dose cohort (5 mg/kg) was expanded to 6 dogs because of a grade 4 neutropenia in the first cycle in one dog. Upon subsequent genetic testing this dog was found to be a heterozygous ABCB1-1Δ mutant, a mutation leading to reduced expression and function of the drug transporter ABCB1/P-glycoprotein, which predisposes to increased toxicity of substrate drugs, including DOX.[44] No other grade 3 or 4 toxicities were encountered in the cohort. One dog in the 7.5 mg/kg HCQ cohort developed grade 4 neutropenia following the first cycle of DOX resulting in expansion of that cohort to 6 patients; this patient was found to be homozygous for the ABCB1-1Δ mutation leading to a complete lack of ABCB1 expression. Of the first 3 dogs enrolled in the 12.5 mg/kg HCQ cohort, 2 treatment-related deaths occurred following the first cycle of DOX and were related to severe neutropenia, gastrointestinal signs (vomiting and diarrhea), and sepsis; one dog developed grade 5 disseminated intravascular coagulation. As the oral HCQ at 12.5 mg/kg was well tolerated and the toxicities were associated with DOX administration, this cohort was expanded with an initial reduction in DOX dose to 25 mg/m². Therefore, 12.5 mg/kg HCQ and 25 mg/m² DOX was determined to be the MTD of the combination. This combination was well tolerated, as 12 dogs were enrolled with only one grade 4 neutropenia and all other toxicities being grade 1 or 2 gastrointestinal effects (**Table 2**).

Responses were evaluated by Response Evaluation Criteria in Solid Tumors[45] and for the 27 dogs with lymphoma, best responses included 22 of 27 (81.5%) complete response, 3 of 27 (11.1%) partial response and 2 of 27 (7.4%) stable disease (**Table 3**). A total of 17 dogs (56.7%) completed 5 cycles of DOX. Of the 13 dogs that did not complete 5 cycles, 7 (53.8%) were due to progressive disease; 2 of these dogs had an initial complete response prior to being removed because of progressive

Table 1. Patient characteristics

Characteristic	Patients (n = 30) No. (%)
Sex	
Male	20 (66.6)
Female	10 (33.3)
Age, y	
Median	7
Range	4–14
Weight, kg	
Median	29.8
Range	8.5–66.5
Breed	
Purebred	18 (59.3)
Mixed	12 (40.7)
Tumor histology	
Lymphoma	27 (88.9)
B-cell	24
T-cell	3
Fibrosarcoma	1 (3.7)
Soft tissue sarcoma	1 (3.7)
Osteosarcoma	1 (3.7)
HCQ dose cohort (mg/kg/d)	
5 mg/kg	6 (20)
7.5 mg/kg	6 (20)
10 mg/kg	3 (10)
12.5 mg/kg	15 (50)
Completed trial	17 (56.7)

disease. In 2 cases, dogs were removed from the trial by owner choice due to perceived reduction in quality of life. The overall median progression-free interval was 5.0 mo, which is similar to single agent DOX when given at 30 mg/m² (**Fig. 1**).[41,42]

Pharmacokinetics

For determination of plasma trough HCQ and N-desethylHCQ levels in the highest dose cohort (12.5 mg/kg), plasma was collected 72 h prior to initiation of therapy and prior to the first dose of DOX. Eleven of the dogs in this cohort had samples available for evaluation. Substantial interindividual variation was noted in these plasma samples and concentrations (mean ± SD) of HCQ and its metabolite were 105.1 ± 73.1 ng/mL and 16.6 ± 5.4 ng/mL, respectively. In 6 of these dogs, plasma was also collected for determination of DOX exposure via a validated limited-sampling method. Predicted plasma DOX exposure ($AUC_{0–6h}$) in these dogs (mean ± SD) was 615.8 ± 208.6 ng/mL. Comparison of the DOX exposure in dogs in this study receiving HCQ followed by DOX at 25 mg/m² with historical controls receiving single agent DOX at 30 mg/m² demonstrated an approximate 25% reduction in AUC (**Table 4**); however, the dose-normalized exposure (AUC divided

Table 2. Hydroxychloroquine and doxorubicin adverse events by HCQ dose cohort

	5 mg/kg (n = 6)	7.5 mg/kg (n = 6)	10 mg/kg (n = 3)	12.5 mg/kg (n = 3)	12.5 mg/kg[a] (n = 12)
HCQ toxicities[b]					
Grade 1/2 toxicities	3 events/ 3 dogs		2 events/ 1 dog	3 events/ 3 dogs	5 events/ 5 dogs
Lethargy	1 (16.7)		1 (33.3)	1 (33.3)	1 (8.33)
Gastrointestinal[§]	2 (33.3)		1 (33.3)	2 (33.3)	4 (25.0)
DOX toxicities					
Grade 1/2 toxicities	10 events/ 5 dogs	4 events/ 3 dogs	3 events/ 2 dogs	1 event/ 1 dog	11 events/ 8 dogs
Lethargy	1 (16.6)				1 (8.33)
Gastrointestinal[c]	5 (83.3)	3 (50.0)	2 (66.7)	1 (33.3)	7 (50.0)
Neutropenia	4 (66.7)		1 (33.3)		3 (16.7)
Thrombocytopenia		1 (16.7)			
Grade 3/4 toxicities	1 event/ 1 dog	6 events/ 3 dogs		2 events/ 2 dogs	5 events/ 2 dogs
Lethargy		1 (16.7)			1 (8.33)
Gastrointestinal		2 (33.3)			2 (16.7)
Neutropenia	1 (16.6)	3 (50.0)		2 (66.7)	1 (8.33)
Thrombocytopenia					1 (8.33)
Grade 5 sepsis				2 (66.7)	
DOX dose reduction	3 (50.0)	3 (50.0)			3 (16.7)

[a]Initial reduction in DOX dose from 30 mg/m² to 25 mg/m². [b]HCQ toxicities were determined as only those occurring during the 72 h administration period prior to the initial dose of DOX. [c]Gastrointestinal toxicities included vomiting, diarrhea, and/or inappetance. Numbers in parenthesis indicate the percentage of dogs in the cohort experiencing toxicity.

Table 3. Treatment efficacy of hydroxychloroquine and doxorubicin in dogs with multicentric lymphoma

	12.5 mg/kg HCQ (n = 15)
Best response	
Complete remission	11 (73.3)[a]
Partial remission	3 (20.0)
Stable disease	1 (6.7)
Progressive disease	0 (0.0)
Overall response rate	93.3
Progression-free interval (mo)	4.9

[a]Numbers in parentheses indicate percent of dogs in the cohort with the response.

by dose) was not significantly different from that observed in dogs receiving single-agent therapy.[46,47] This suggests no pharmacokinetic interaction between HCQ and DOX that would result in altered plasma DOX exposure.

Tumor tissue concentrations of HCQ and its metabolite were also determined in the 11 dogs in which plasma concentrations were available. Results indicate a significant accumulation of HCQ and the metabolite in tumor tissues, with an approximate 100-fold increase in tumor compared with plasma (**Fig. 2A**). Although tumor tissue concentrations were consistently higher, there was no significant correlation between the plasma and tumor concentration for individual dogs (Pearson correlation = 0.143, *P* = 0.695, **Fig. 2A**).

Pharmacodynamic response in peripheral blood and tumor tissue

The pharmacodynamic response to HCQ was evaluated in peripheral blood mononuclear cells (PBMCs) through flow cytometric evaluation of changes in LC3 for 6 dogs treated at 12.5 mg/kg po qd HCQ/25 mg/m2 DOX. All 6 dogs had a complete response and minimal toxicity (**Table 4**). Comparison of pre- and post-treatment mean fluorescence intensity (MFI) for LC3 by flow cytometry in PBMC revealed a significant increase of nearly 2-fold (*P* = 0.033) (**Fig. 2C**). Additionally, we employed the gold standard, electron microscopy (EM), to visualize the formation and accumulation of autophagosomes, which is indicative of a blockade in the autophagic pathway by a drug such as HCQ, which alters the lysosomal pH and thus prevents fusion with autophagosomes leading to increased accumulation of autophagosomes (**Fig. 2D**).[48] In tumor tissues, the pharmacodynamic response was evaluated by western analysis for changes in LC3 and SQSTM1. All biopsies were from involved lymph nodes in patients with NHL in the 12.5 mg/kg dose cohort. Though not significant, there was a trend toward increases in LC3-II and SQSTM1 expression in biopsy samples as well as tissue aspirates following HCQ administration (**Fig. 2E and F**). Taken together, these data indicate that a variety of pharmacodynamic assays,
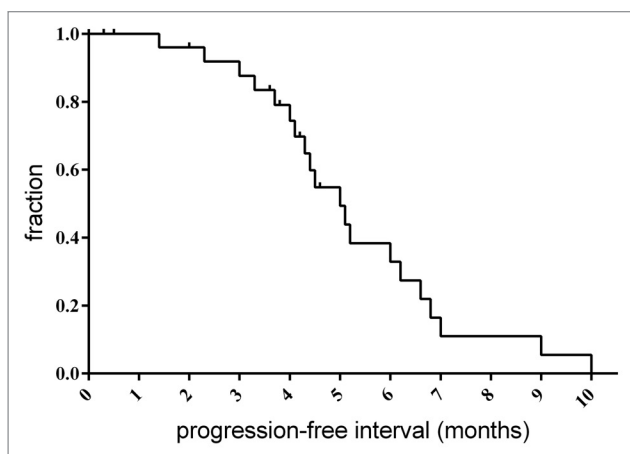

Figure 1. Progression-free interval. Progression-free interval was determined for the 27 lymphoma patients receiving the HCQ and DOX combination. Median time to progression was 4.9 mo.

Table 4. Comparison of predicted and dose-normalized doxorubicin exposure between dogs administered hydroxychloroquine at 12.5 mg/kg daily and historical controls receiving single agent doxorubicin

Parameter	Current study (n = 12) mean ± SD	Historical controls (n = 27) mean ± SD
DOX_{5min} (ng/mL)	492.2 ± 250.1	411.97 ± 166.3
DOX_{45min} (ng/mL)	35.8 ± 7.5	69.2 ± 24.4
$DOX_{60\,min}$ (ng/mL)	28.45 ± 8.5	57.9 ± 12.1
Pred AUC_{0-6h} (ng*mL/hr)	615.8 ± 208.6	825.6 ± 176.9[a]
Dose-normalized exposure (ng*mL/hr)/(mg/m²)	24.6 ± 8.3	27.5 ± 5.9

[a]Significant difference in AUC_{0-6h} between study subjects receiving 25 mg/m² doxorubicin and historical controls receiving doxorubicin alone at 30 mg/m²; 2-tailed t test, $P = 0.016$. Difference in dose-normalized exposure is not significant between study subjects and historical controls.
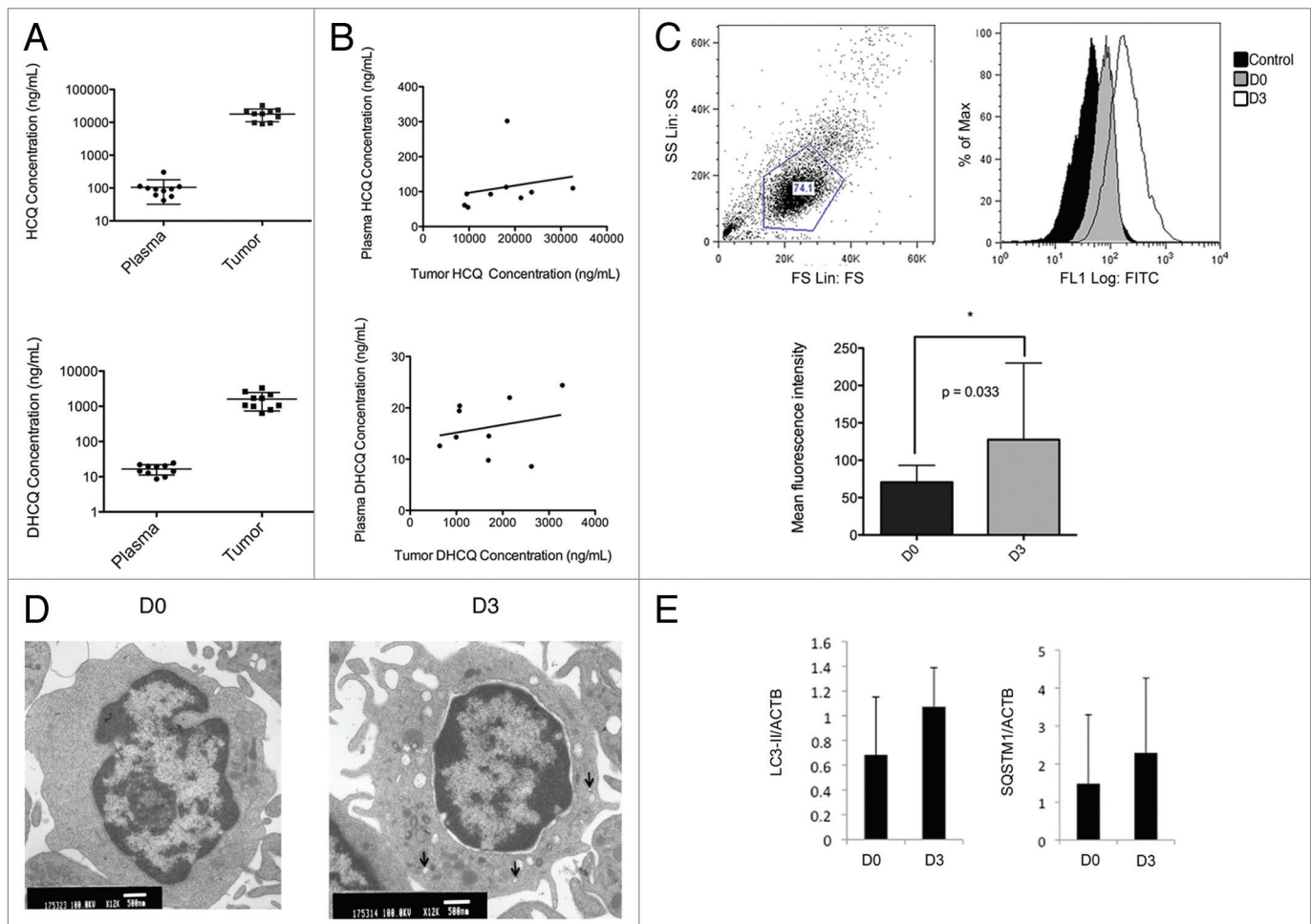

Figure 2. Assessment of pharmacodyanmic response. Concentrations of hydroxychloroquine (HCQ) and the metabolite N-desethylhydroxychloroquin (DHCQ) in dogs administered 12.5 mg/kg HCQ daily were significantly higher in tumor tissues compared with plasma [HCQ ($P < 0.0001$) and DHCQ ($P = 0.0003$)] (**A**). There was no significant correlation between plasma and tumor HCQ or DHCQ concentrations (Pearson correlation = 0.143 and 0.238, $P = 0.695$ and 0.537) (**B**). PBMCs were isolated from whole blood before and 3 d post HCQ administration. The representative plot shows gating of cells and histograms demonstrate the increase of LC3 positive cells after HCQ administration (**C**). Overall there was a significant increase in the mean fluorescence intensity post HCQ. Representative EM images of PBMCs taken pre and post HCQ administration. There is an increase in autophagic vesicles after HCQ treatment, indicated by arrows (**D**). Tumor biopsies were taken before and 3 d post HCQ administration. Western blot analysis was performed on biopsies to determine expression of LC3-II. Though not significant, both increased overall after treatment (**E**).

that are feasible in the clinical setting, show strong evidence of autophagy inhibition in PBMCs and also in tumor tissue. It should however be noted that in the tumor tissue these responses were less robust than in the blood.

Discussion

Autophagy inhibition is thought to be a mechanism of survival and drug resistance for many types of cancers. Thus combining the autophagy inhibitor HCQ with cytotoxic chemotherapy may enhance efficacy. Here we report the results of a phase I/II clinical trial evaluating the use of combined HCQ and DOX in dogs with spontaneously occurring cancer, aimed at defining a safe and potentially biologically effective dose of HCQ. The rationale for the dosing scheme of 72 h pretreatment prior to the first dose of DOX followed by continuous daily dosing with HCQ was based on the reported long half-life and time to reach steady-state in humans, and the lack of any corresponding pharmacokinetic data in dogs.[19-21] Four dose cohorts, ranging from 5 mg/kg/d up to 12.5 mg/kg/d, were evaluated. This study identified maximum tolerated doses of HCQ in combination with DOX of 12.5 mg/kg/d, and 25 mg/m², respectively. Consistent with previous reports of the clinical use of HCQ in dogs, toxicities attributable to HCQ alone were generally mild with the most commonly reported adverse events being grade 1 lethargy and gastrointestinal disturbance.[20,21] Dose-limiting toxicities following DOX administration were observed in 1 dog in each of the 5 mg/kg and 7.5 mg/kg dose cohorts; however, the finding that both of these dogs were carriers of the 1Δ mutation of the *ABCB1/MDR1* gene (termed *Abcb1a* in mice and rats), would support the argument that these toxicities were not due to the combination of HCQ and DOX but an intrinsic sensitivity to DOX because of reduced or absent function of the ABCB1 efflux pump. Our finding of 12.5 mg/kg/d as the MTD of HCQ is based on the necessity to reduce the DOX dose by approximately 20% (30 mg/m² to 25 mg/m²) in order to avoid the grade 5 adverse events that occurred at the standard DOX dose. Twelve dogs with lymphoma were subsequently enrolled in the 12.5 mg/kg/d HCQ and 25 mg/m² DOX cohort with 2 requiring an additional 20% dose reduction in DOX due to grade 4 neutropenia. Importantly, 9 of these dogs achieved a complete remission and 8 of these dogs completed the trial, which would indicate that the reduced DOX dose did not result in a reduction in initial response. We did observe a substantial overall response rate in dogs with lymphoma (93.3%). These results are encouraging given that reported response rates with DOX alone for treatment-naive lymphoma in dogs range from 60% to 85%.[41,42] The median progression-free interval in dogs with lymphoma in this study was 5.0 mo which is comparable to single agent DOX at 30 mg/m².[41,42] A larger study group with longer follow-up would be required to determine if the combined HCQ and lower DOX (25 mg/m²) dose is superior, or at least as effective, as single agent DOX administered at the standard 30 mg/m² dose.

We were able to evaluate plasma exposure (area under the concentration-time curve) of DOX in 6 dogs within the highest HCQ dose cohort. Samples from this cohort were chosen as it was assumed that if changes in DOX PK were to be seen it would most likely occur with the highest HCQ dose. This was evaluated with the use of a validated limited-sampling model to predict overall exposure to DOX. We were able to demonstrate that overall plasma DOX exposure (dose-normalized area under the plasma concentration-time curve) was not significantly different in our group from dogs receiving DOX alone.[46,47]

In our study we were able to detect pharmacodynamic activity in PBMC and in tumor tissue following 72 h of oral HCQ administration, although the extent of autophagy inhibition, at least as determined by analysis of LC3, was less robust and at a lower level of statistical significance in tumor tissue compared with blood. Importantly, we found significant accumulation of HCQ in tumor tissue relative to plasma. A substantial fraction of HCQ partitions to red blood cells, and it is possible that whole blood levels would correlate with concentrations in homogenized tumor especially since the tumor samples also include some blood cells in them and do not reflect only tumor cells; however, this seems unlikely as 4-aminoquinolone binding to red blood cells has been reported to be linear with concentration and red blood cell density.[49] This infers a constant relationship between total concentration and the fraction bound to red blood cells, and while whole blood levels are certain to be higher than plasma, the same relationship to tumor concentrations would hold true. It is important to note that this study also evaluated plasma HCQ concentrations after 72 h of therapy and, based on the reported long half-life in humans (see Rosenfeld et al., this issue[50]; Vogl et al., this issue[51]; Rangwala et al., this issue[52,53]), it is possible samples were taken before dogs achieved steady-state concentrations. Variability and delays in achieving steady-state concentrations have been reported to contribute to the variability in tissue PD response in humans.[17] However, it does appear that plasma HCQ concentration is not an adequate surrogate for concentrations within the tumor and, similarly, evidence of autophagy inhibition in PBMCs is not necessarily sufficient to infer that autophagy was effectively inhibited in the tumor. These data suggest that although HCQ is capable of inhibiting autophagy in tumors of cancer patients, autophagy inhibitors with better pharmacokinetics and tumor bioavailability would be useful. Additionally our results suggest that one should be cautious in inferring from surrogate markers in the blood that sufficient tumor drug levels for effective autophagy inhibition have been achieved and emphasize the value in future clinical trials of attempting to make such measurements in tumor tissue.

In conclusion, this is the first study to evaluate the safety and potential clinical utility of autophagy inhibition using HCQ combined with cytotoxic chemotherapy in dogs with spontaneous cancer. We used continuous oral administration of HCQ combined with DOX on a 21-d cycle and showed that HCQ at doses up to 12.5 mg/kg/d are well tolerated but necessitate a reduction in the standard dose of DOX used in order to avoid unacceptable toxicity. Importantly, this reduction in DOX still provided a superior ORR and comparable PFI to dogs receiving

single-agent, standard dose DOX. We were able to show target modulation in both PBMC and tumor tissue. The superior overall response rate and comparable progression-free interval in our study provide strong support for further evaluation of this combination in randomized, placebo-controlled studies in canine lymphoma, and the strength of the canine model of NHL supports initiation of similar clinical trials in human lymphoma patients.

Materials and Methods

Patient recruitment

All dogs in this study were client-owned, pet dogs presenting to the Colorado State University Flint Animal Cancer Center. Study participation was offered in cases where standard therapy had been declined by the dog's owner or such therapy had previously failed, or in cases of advanced disease where no meaningful standard therapy exists. Protocol approval was obtained from the Institutional Animal Care and Use Committee and the Colorado State University Veterinary Teaching Hospital Clinical Review Board. All dogs were treated in accordance with the NIH Guidelines for Care and Use of Laboratory Animals. Signed informed consent and consent to necropsy were obtained from all owners prior to enrollment in the study.

This study was initially open to dogs with histologically or cytologically confirmed neoplasia of any histotype for which single-agent DOX would be an acceptable therapy. Dogs with regional or distant metastasis or locally advanced disease were included if a survival time of > 6 wk was anticipated. Dogs were required to be free of other severe complicating concurrent disease conditions, and were required to have adequate clinical indices to safely undergo chemotherapy and, in some cases, sedation for tumor biopsy acquisition (specifically, total bilirubin not exceeding 1.5× normal; creatinine no exceeding 2× normal; at least 2,500 neutrophils/μL, 75,000 platelets/μL, and a hematocrit of at least 28%). A Veterinary Comparative Oncology Group performance status of 0 or 1 was required for study inclusion (0, normal activity; 1, restricted activity [decreased from predisease status]; 2, compromised [ambulatory only for vital activities, consistently defecates and urinates in acceptable areas]; 3, disabled [requires force feeding, is unable to confine urination and defecation to acceptable areas]; 4, dead). Prior chemotherapy and radiation therapy were allowed with a 3- and 6-wk washout period, respectively. If prednisone was utilized as an anti-neoplastic agent, a 72 h washout was required and no concurrent antineoplastic therapy was allowed. For dogs previously administered DOX, prior cumulative exposure could not exceed 90 mg/m^2.

Pretreatment procedures and evaluations

A complete blood count (CBC), serum biochemistry profile, and urinalysis were done prior to enrollment. Staging and immunophenotyping were performed as appropriate for the specific tumor type. Heparinized whole blood (10 to 12 mL) was collected for separation of peripheral blood mononuclear cells (PMBC), and a 14-gauge needle core biopsy was obtained from accessible tumors using local anesthesia or brief sedation, as necessary.

Treatments

All dogs were given oral HCQ sulfate tablets (Ranbaxy Pharmaceuticals Inc., NDC 63304-0296-01) once daily, beginning 72 h prior to DOX (Pfizer Inc., NDC 0069-3034-20) administration and continuing through the remainder of the study. An initial dose of 5 mg/kg was chosen as the starting point and doses were escalated according to a standard 3 × 3 dose-escalation protocol whereby 3 dogs were enrolled in each dose cohort and the cohort expanded to 6 if dose limiting toxicity (grade 3 or higher) was encountered in 1 of the first 3 dogs. Dogs were scheduled to receive a standard dose of DOX (30 mg/m^2 intravenously, or 1 mg/kg if < 15 kg) as initial treatment on d 4 and continued on a 21-d cycle for a maximum of 5 treatments or disease progression. As with single agent DOX, dose reductions of 20% were instituted for subsequent treatments if grade 3 or 4 toxicities were observed after the first dose.

Monitoring procedures and evaluations

Adverse events were recorded on d 4, 11, and at each subsequent visit. All treatment-related adverse events were graded based upon the guidelines set forth in the Veterinary Comparative Oncology Group-Common Terminology Criteria for adverse events v1.0.[54] A CBC and blood chemistry were obtained 72 h after initiation of HCQ therapy. Serum, plasma, and heparinized whole blood were collected prior to DOX administration on d 4 for determination of trough HCQ and DHCQ levels and evaluation of HCQ pharmacodynamics in blood. For 6 dogs in the highest HCQ dose cohort (12.5 mg/m^2), plasma was obtained at 5, 45, and 60 min for evaluation of DOX exposure utilizing a previously published limited-sampling model.[55] Tumor biopsies were obtained following the initial 72 h therapy with HCQ for determination of HCQ and DHCQ levels and pharmacodynamics. A CBC and blood chemistry were obtained 7 and 21 d following DOX administration. Owners were asked to fill out quality of life/pain questionnaires prior to the study, after the initial 72 h of HCQ therapy, and again at each subsequent visit. The HCQ-DOX combination was continued on an every-3-wk basis until disease progression, maximal cumulative DOX dosage (> 150 mg/m^2), or owner request. Tumor responses were evaluated using Response Evaluation Criteria in Solid Tumors criteria[45] on measured lymph nodes.

PBMC Isolation

Twenty mL of whole blood were collected from dogs and divided. Peripheral blood mononuclear cells (PBMC) were isolated using lymphocyte separation media (Cellgro, 25-072-CV). Briefly, one volume of phosphate-buffered saline containing 5 mmol/L EDTA was added to heparinized blood samples, which were then underlaid with 2 mL of lymphocyte separation media. The samples were centrifuged at 400 × g for 20 min and the lymphocyte layer was aspirated and washed 3 times in one volume of PBS/EDTA. Isolated PBMCs were stored at –80 °C until processing for flow cytometry, or EM analysis.

Flow cytometry

After PBMCs were isolated, cells were immediately resuspended in 200 μL of FACs buffer (2% FBS and 0.05% sodium

azide in phosphate buffered saline). Cells were centrifuged at 700 × g for 2 min and then fixed and permeabilized for 18 h at 4 °C in diluted Fix/Perm buffer (eBiosciences, 00-5123-43 and 00-5223-56). Cells were washed once in diluted Perm Buffer (eBiosciences, 00-8333-56) and then stained with anti-LC3 (Novus Biologicals, NB100-2220) at 1:40 for 30 min at room temperature. Two wash steps were done in Perm Buffer and cells were stained with anti-rabbit secondary conjugated to FITC (Bethyl Laboratories, A120-101F) at 1:40 for 30 min at room temperature then washed twice with FACs buffer. Cells were then analyzed by flow cytometry using a Cyan cytometer (DakoCytomation, Carpenteria, CA) with Summit version 4.3.02 and FlowJo version 7 analysis software. The MFI between pre- and post-treatment with HCQ was used to compare samples for evidence of autophagy inhibition.

Electron microscopy

PBMC samples were fixed in Karnovsky fixative (3% glutaraldehyde, 2% formaldehyde, 0.1 M sodium phosphate buffer, pH 7.4; Electron Microscopy Sciences, 15720) and stored at 4 °C until processing. Following fixation, samples were washed 3 × 10 min with buffer, and postfixed for 1 h with 1% osmium tetroxide in 0.1 M sodium phosphatebuffer. After osmication, samples were again washed 3 times with buffer, dehydrated through a graded ethanol series (10 min each in 50%, 70%, 80%, and 90% ethanol, 2 × 10 min in 100% ethanol), transferred to propylene oxide (10 min in 1:1 ethanol:propylene oxide, 2 × 10 min in 100% propylene oxide), and infiltrated with Eponate 12 resin (medium hardness 45% glycerol polyglycidyl, 30.5% dodecenyl succinic anhydride, 23% NMA methylnadic anhydride, and 1.5% DMP-30; Ted Pella, Inc., 18010). Resin-embedded samples were polymerized for 24 h at 65 °C. Ultrathin sections 60 to 90 nm in thickness were cut from the embedded samples using a Diatome diamond knife and a Reichert Ultracut E ultramicrotome, mounted on formvar-coated slot grids (Electron Microscopy Sciences, 2010-Cu), and poststained with uranyl acetate and lead citrate. Sections were examined and photographed at 12,000× using a JEOL JEM-2000EX II electron microscope (JEOL USA, Inc., Peabody MA) operated at 100 kV. Negatives were scanned at 1200 ppi using an Epson Perfection flatbed scanner (Epson America, Inc., Long Beach, CA).

Western blot analysis

For protein extraction from tumors, snap-frozen biopsies were placed in 500 μL of lysis buffer (0.01% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.5, 0.2 mM Na-orthovanadate [Alexis Biochemicals, 400-032-G025], 34.8 μg/mL PMSF [Fluka Biochemica, 78830], and 1× protease inhibitor cocktail [Roche, 11836153001]). Samples were then homogenized for 20 s on ice and sonicated on ice for 3, 3 s pulses. Samples were centrifuged at 21,000 × g for 5 min at 4 °C and supernatant fractions collected.

Protein concentration was determined using a bicinchoninic acid protein assay (Thermo Scientific, 23225). Thirty micrograms of protein was used in SDS-PAGE and transferred onto PVDF membranes (Millipore, IPVH0010). Blots were blocked in 2.5% nonfat dry milk in Tris-buffered saline/Tween 80 (10 mM Tris pH 7.5, 100 mM NaCl, and 0.2% Tween 80; Fisher

Chemicals, BP338-500) for 1 h at room temperature. Blots were probed with anti-LC3 (Novus Biologicals, NB100-2220), at 1:1000, anti-SQSTM1 (Abnova, H0008878-M01), at 1:1000, or anti-ACTB/β-actin (Sigma, A5441), at 1:5000, antibodies were incubated overnight at 4 °C. After 3 washes in TBST, membranes were incubated for 1 h at room temperature with either anti-rabbit (Pierce, 31460) or anti-mouse (Pierce, 31430) secondary antibodies conjugated to HRP. Immunoreactive proteins were detected using West Dura (Thermo Scientific, 37071) and imaged in a ChemiDoc XRS+ (Bio Rad, Hercules, CA) using Image Labs version 3.0 software. Densitometry analysis was performed using ImageJ software available online from the NIH (http://rsb.info.nih.gov/ij/index.html). LC3-I (cytosolic form of LC3) or LC3-II (autophagosome-associated) and SQSTM1 were first normalized to actin loading controls and then relative density determined for post HCQ administration samples by comparison to preadministration samples. A relative density of 1.5 in post-treatment samples was chosen as the cut-off for evidence of autophagy inhibition.

Hydroxychloroquine analysis in plasma and tumor tissue by liquid chromatography-tandem mass spectrometry

Hydroxychloroquine and the main metabolite DHCQ were measured in dog plasma and tissue biopsies using a liquid chromatography/tandem mass spectrometry assay using chloroquine as an internal standard. Positive ion electrospray ionization mass spectra were obtained with a MDS Sciex 3200 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Inc., Foster City, CA) with a turbo ionspray source interfaced to Shimadzu LC-20AD Series Binary Pump HPLC system (Columbia, MD). Samples were chromatographed with a Sunfire 2.5 μm, C8, 4.6 × 50 mm column. A liquid chromatography gradient was employed with mobile phase A consisting of 20 mM ammonium acetate containing 0.5% acetic acid and mobile phase B consisting of acetonitrile containing 0.5% acetic acid at 800 μL/min. Chromatographic separation was achieved by holding mobile phase B steady at 2% from 0 to 0.25 min, increasing linearly to 95% at 3 min, holding mobile phase B steady at 95% from 3.0 to 3.5 min, decreasing linearly to 2% at 3.75 min, followed by re-equilibration at 2% B until 4.5 min. The sample injection volume was 10 μL and the analysis run time was 4.5 min. The mass spectrometer settings were optimized as follows: turbo ionspray temperature, 550 °C; ion spray voltage, 1500 V; source gas 1, 60 units; source gas 2, 50 units; curtain gas, 45; collision gas, medium. Compound parameters for HCQ were optimized as follows: declustering potential, 47.9 V; entrance potential, 4.30 V; collision cell entrance potential, 14.0 V; collision energy, 29.7 V; collision cell exit potential 3.9 V. Sample concentrations of HCQ and metabolite were quantified by internal standard reference method in the multiple reaction monitoring mode with ion transitions m/z 336.2 → 247.1 atomic mass units (amu) for HCQ, m/z 308.2 → 179.0 and 308.2 → 130.1 amu (summed) for N-DHCQ, and m/z 320.3 → 247.1 amu for the internal standard, CQ. Scan times were 200 ms, and Q1 and Q3 were both operated in unit resolution mode.

Analytical standards (1 ng to 1,000 ng/mL), quality control (5, 50, and 500 ng/mL), and unknown plasma samples were

prepared via a liquid-liquid extraction method whereby 180 μL of unknown or fortified plasma samples were added to 1.5 mL polypropylene tubes containing 50 ng of internal standard (CQ) followed by 500 μL of ethyl acetate. Samples were then vortex mixed for 8 min and centrifuged at 21,000 × g for 8 min. The organic phase was transferred to fresh microcentrifuge tubes and evaporated to dryness. Samples and standards were then reconstituted in 200 μL of a 1:1 methanol/20 mM ammonium acetate mixture at pH 4.0 and transferred to autosampler vials containing glass inserts. For analysis in tissue, biopsy samples were homogenized in Milli-Q H_2O at a concentration of 100 mg/mL and 180 μL of homogenate was prepared as described above. The lower limit of quantification for HCQ was 5 ng/mL, and for DHCQ it was 10 ng/mL. The standard curves were linear across the range of concentrations utilized. Accuracy of the standard curve and the quality control samples was within 15% at all concentrations and precision was within 15% of the coefficient of variation.

Doxorubicin, doxorubicinol, and aglycone analysis in plasma by liquid chromatography-tandem mass spectrometry

Doxorubicin and its metabolites, doxorubicinol and doxorubicin aglycone, were measured in canine plasma using a liquid chromatography/tandem mass spectrometry assay. Negative ion electrospray ionization mass spectra were obtained with the instrumentation described above. Samples were chromatographed on a Waters Sunfire 5 μm, C8 column (4.6 × 50 mm) (Waters Corporation, Milford, MA) with a Phenomenex C18 filter frit guard cartridge (Torrance, CA). A liquid chromatography gradient was employed with mobile phase A consisting of 10 mM ammonium acetate containing 0.1% formic acid and mobile phase B consisting of methanol at 1200 μL/min. Chromatographic separation was achieved by holding mobile phase B steady at 25% from 0 to 1.0 min, increasing linearly to 98% at 2 min, holding mobile phase B steady at 98% from 2.0 to 3.0 min, decreasing linearly to 25% at 4.0 min, followed by re-equilibration at 25% B until 4.5 min. The sample injection volume was 60 μL and the analysis run time was 4.5 min. The mass spectrometer settings were optimized as follows: turbo ionspray temperature, 575 °C; ion spray voltage, –4500 V; source gas 1, 60 units; source gas 2, 60 units; curtain gas, 10; collision gas, low. Compound parameters for DOX were optimized as follows: declustering potential, –41.7 V; entrance potential, –6.3 V; collision cell entrance potential, –15.9 V; collision energy, –22.5 V; collision cell exit potential –3.9 V. Sample concentrations of DOX and metabolite were quantified by internal standard reference method in the multiple reaction monitoring mode with ion transitions m/z 542.3 → 395.4 amu for DOX, m/z 544.3 → 397.4 and 544.3 → 309.5 amu (summed) for doxorubicinol, and m/z 526.2 → 379.3 amu for the internal standard, daunorubicin (Sigma, 30450). Scan times were 200 ms, and Q1 and Q3 were both operated in unit resolution mode. Analytical

standards (1 to 1,000 ng/mL), quality control (5, 100, and 500 ng/mL), and unknown plasma samples were prepared via a liquid-liquid extraction method whereby 100 μL of unknown or fortified plasma samples were added to 1.5 mL polypropylene tubes containing 100 ng/mL of internal standard (daunorubicin) followed by 1,000 μL of ethyl acetate. Samples were then vortex mixed for 10 min and centrifuged at 17,000 × g for 10 min. The organic phase (950 μL) was transferred to fresh microcentrifuge tubes and evaporated to dryness. Samples and standards were then reconstituted in 100 μL of 1:1 methanol/10 mM ammonium acetate with 0.1% formic acid and transferred to autosampler vials containing polypropylene inserts.

Prediction of doxorubicin exposure by limited sampling

Six dogs in the 12.5 mg/kg dose cohort had plasma samples collected at 5, 45, and 60 min following the first dose of DOX. Plasma DOX concentrations at these time points were used to predict the overall DOX exposure (AUC) using a limited sampling method that was previously validated and published by the authors.[40] In this model, overall DOX exposure was predicted using the equation:

$$AUC = 46.9 + 0.63 \cdot C_{5min} + 1.96 \cdot C_{45min} + 6.63 \cdot C_{60min}$$

where $C_{5min, 45min, 60min}$ refer to plasma DOX concentrations at those time points. These AUC values were dose-normalized and compared with historical control data generated in our laboratory for dogs receiving DOX alone to evaluate for potential interactions between HCQ and doxorubicin that might result in alterations in overall exposure.[47]

Statistical analysis

All statistical analysis was performed in Prism version 5.0 (GraphPad Software). A paired, 2-tailed Student t test was used to compare average MFI pre- and post-HCQ administration in PBMCs and lymph node aspirates. An unpaired, 2-tailed Student t test was used to compare DOX exposure between study subjects and historical controls. Pearson correlation was performed to determine correlations between pharmacokinetic and pharmacodynamic endpoints. A P value of less than 0.05 was considered significant.

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