Cancer Therapy: Preclinical

Phase I Pharmacokinetic and Pharmacodynamic Evaluation of Combined Valproic Acid/Doxorubicin Treatment in Dogs with Spontaneous Cancer

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Abstract

Purpose: Histone deacetylase inhibitors (HDACi) are targeted anticancer agents with a well-documented ability to act synergistically with cytotoxic agents. We recently showed that the HDACi valproic acid sensitizes osteosarcoma cells to doxorubicin in vitro and in vivo. As there are no published reports on the clinical utility of HDACi in dogs with spontaneous cancers, we sought to determine a safe and biologically effective dose of valproic acid administered prior to a standard dose of doxorubicin.

Methods: Twenty-one dogs were enrolled into eight cohorts in an accelerated dose-escalation trial consisting of pretreatment with oral valproic acid followed by doxorubicin on a three-week cycle. Blood and tumor tissue were collected for determination of serum valproic acid concentration and evaluation of pharmacodynamic effects by immunofluorescence cytochemistry and immunohistochemistry. Serum and complete blood counts were obtained for determination of changes in doxorubicin pharmacokinetics or hematologic effects.

Results: All doses of valproic acid were well tolerated. Serum valproic acid concentrations increased linearly with dose. Doxorubicin pharmacokinetics were comparable with those in dogs receiving doxorubicin alone. A positive correlation was detected between valproic acid dose and histone hyperacetylation in peripheral blood mononuclear cells. No potentiation of doxorubicin-induced myelosuppression was observed. Histone hyperacetylation was documented in tumor and peripheral blood mononuclear cells. Responses included 2 of 21 complete, 3 of 21 partial, 5 of 21 stable disease, and 11 of 21 progressive disease.

Conclusions: Valproic acid can be administered to dogs at doses up to 240 mg/kg/day prior to a standard dose of doxorubicin. In addition, we have developed the pharmacokinetic/pharmacodynamic tools necessary for future studies of novel HDACi in the clinical setting of canine cancer.

Histone deacetylase (HDAC) enzymes, responsible for the removal of acetyl groups from the NH2-terminal tails of histone proteins, play a crucial role in chromatin plasticity and have recently been identified as one of the most promising therapeutic targets in cancer therapy (1). The rationale behind the use of HDAC inhibitors (HDACi) stems from the discovery of epigenetically silenced tumor suppressor genes in a number of model systems resulting in tumor cell addiction to altered signaling pathways (2, 3). In addition, an imbalance in the activity of histone acetyltransferases, the enzymes responsible for lysine acetylation, and HDAC plays a crucial role in the development and progression of some tumors (4). A number of HDACi have shown antitumor activity in vitro and in vivo, either as single agents or in combination with other chemotherapeutics, and have shown efficacy in hematologic and solid tumors through inhibition of proliferation by cell cycle arrest, effects on terminal differentiation, and apoptosis (5–13). A few of these agents have entered into clinical trials, and of the HDACi, suberoylanilide hydroxamic acid (Vorinostat) and romidepsin (Istodax) have received Food and Drug Administration approval for use in humans as single agents in the treatment of cutaneous T-cell lymphoma. For the most part, these agents are well tolerated and show target modulation in tumor tissues as well as some single-agent efficacy in solid and hematologic malignancies (8, 14, 15). However, these agents are likely to provide the most benefit when combined with other treatment modalities such as chemotherapy or radiotherapy.

Although early-phase studies in humans provide promising results and describe some biological parameters for
determining potential susceptibility to HDACi-containing treatment protocols, it has also been clearly shown that the current drug development pipeline for novel anticancer therapies in many cases fails to properly identify agents that provide long-term improvements in patient survival. The use of spontaneously occurring tumors in pet dogs has the potential to improve current drug development and modeling strategies (16–18). This realization has led to the completion and publication of the first canine clinical trial conducted by the National Cancer Institute’s Comparative Oncology Trials Consortium evaluating the safety, toxicity, tumor specificity, and efficacy of a novel tumor endothelium targeting agent (RGD-A-TNF) being developed as a therapy for human cancers (18). The translational utility of the canine cancer model stems from the greater similarities to human cancers when compared with murine models. Unlike murine models, dogs are relatively outbred, immunocompetent animals with spontaneously occurring tumors experiencing spontaneous metastasis and therapy resistance, representing a spectrum of tumor histotypes that have biology similar to that found in humans. When compared with murine tumors, the relatively large size of canine tumors more closely approximates human solid tumors with respect to important biological factors such as hypoxia and clonal variation, and allows for multiple samplings of tumor tissue over time. The relatively rapid time course of disease progression, when compared with human cancer, allows for more rapid assessment of therapeutic end points than is possible in many human clinical trials (19, 20). In addition, the lack of a meaningful standard of care for many types of canine cancer alleviates the obligation to start with single-agent phase I trials and allows immediate evaluation of novel agents in combination protocols, where they are most likely to show a benefit.

Although there have been some reports on the in vitro sensitivity of canine cancer cells to HDACi (21, 22), there have been no reports on the clinical use of HDACi in canine cancer patients.

The antiepileptic drug valproic acid, belonging to the short-chain fatty acid class of HDACi, has shown efficacy in a variety of tumor models, especially when combined with other forms of therapy (5, 6, 15, 23–26). We had previously shown that pretreatment of canine and human osteosarcoma cells with valproic acid results in sensitization to doxorubicin, partially via increasing nuclear doxorubicin accumulation, resulting in decreased proliferation and increased apoptosis (22). This was also shown in an in vivo xenograft model of canine osteosarcoma (22). Here we report the pharmacokinetic and pharmacodynamic results of a phase I trial of oral valproic acid in tumor-bearing dogs given prior to a standard dose of doxorubicin. We hypothesized that valproic acid could be safely administered to dogs for 48 hours prior to a standard dose of doxorubicin, would show target modulation in peripheral blood mononuclear cells (PBMC) and tumor tissues, and would have no effects on doxorubicin pharmacokinetics.

Materials and Methods

Chemicals and antibodies

Divalproex sodium extended-release tablets (Depakote ER) were purchased from Cardinal Health. Doxorubicin was purchased from Bedford Laboratories. Controls for the valproic acid assay were purchased from Cliniqa. Anti–acetyl-histone H3 and H4 and total histone H3 and H4 antibodies were purchased from Upstate Biotechnology. FITC-conjugated goat anti-rabbit antibody was purchased from Bethyl Laboratories. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody was purchased from Pierce. Daunorubicin hydrochloride was purchased from Sigma, and doxorubicinol hydrochloride from Toronto Research Chemicals, Inc. All other reagents were of analytical grade.

Patient recruitment

All dogs in this study were pet dogs presenting as patients to the Colorado State University Animal Cancer Center. Study participation was offered in cases where standard therapy had failed or had been declined by the dog’s owner, or in cases of advanced disease where no meaningful standard therapy exists. Dogs were treated in accordance with the NIH Guidelines for Care and Use of Laboratory Animals. Protocol approval was obtained from the Institutional Animal Care and Use Committee and the Colorado State University Veterinary Teaching Hospital Clinical Review Board. Signed informed consent and consent to necropsy were obtained from all owners.
This study was open to dogs with histologically or cytologically confirmed neoplasia. Dogs with regional or distant metastasis or advanced local disease were included if a survival time of >6 weeks was expected. Dogs were required to be free of other severe complicating concurrent disease conditions, and were required to have adequate laboratory and clinical indices to safely undergo therapy (specifically, total bilirubin not exceeding 1.5× normal; creatinine not exceeding 2× normal; at least 2,500 neutrophils/μL, 75,000 platelets/μL, and a hematocrit of at least 28%). Treatment-related adverse events were graded based on guidelines set forth in the Veterinary Comparative Oncology Group-Common Terminology Criteria for Adverse Events (VCOG-CTECAE; ref. 27). A VCOG performance status of 0 to 1 was required for study inclusion [0, normal activity; 1, restricted activity (decreased activity from predisease status); 2, compromised (ambulatory only for vital activities, consistently defecates and urinates in acceptable areas); 3, disabled (dog needs to be force-fed, is unable to confine urination and defecation to acceptable areas); 4, dead]. Prior chemotherapy and radiation therapy were allowed with a 3-week or 6-week washout period, respectively. In addition, a 72-hour washout from prednisone was required if being used as an antineoplastic drug. No concurrent antineoplastic therapy was allowed, and prior doxorubicin exposure could not exceed 90 mg/m².

#### Pretreatment procedures and evaluations

A complete blood count (CBC), serum biochemistry profile, and urinalysis were done prior to enrollment in the study, and staging was done as appropriate for specific tumor type. Heparinized whole blood (10 mL) was collected for PBMC separation, and 14-Ga needle core biopsies were obtained from accessible tumors using local anesthesia or brief sedation.

#### Treatments

All dogs were given oral valproic acid for 48 hours prior to doxorubicin administration with an initial loading dose of 2× the intended maintenance dose, and two additional doses were administered on the day of doxorubicin administration (total of 6 doses, or 72 hours of therapy). The initial maintenance dose of 30 mg/kg was chosen as the starting point as this is one half of the putative anticonvulsant dose for valproic acid in dogs (28). Doses were escalated according to an accelerated dose-escalation protocol whereby one dog was enrolled in each cohort and the cohort was expanded to six only if toxicity (grade 3 or higher) was encountered in the first dog. All dogs received a standard dose of doxorubicin (30 mg/m², or 1 mg/kg if <15 kg) as initial treatment on day 3, between the morning and evening valproic acid doses. Doxorubicin dose was reduced by 20% for subsequent treatments if grade 3 or 4 toxicities were observed after the first dose.

#### Monitoring procedures and evaluations

A CBC and blood chemistry were obtained 48 hours after initiation of valproic acid therapy. Serum, plasma, and heparinized whole blood were collected immediately prior to the morning valproic acid dose on day 3 for determination of valproic acid trough concentrations and valproic acid pharmacodynamic evaluation. Serum was collected after doxorubicin administration at 0, 10, 20, 30, 60, 120, 240, 360 minutes, and 24 hours for evaluation of doxorubicin pharmacokinetics. Tumor biopsies were obtained again 48 hours after initiation of valproic acid therapy. A CBC and blood chemistry were obtained 7 and 21 days following doxorubicin administration. Owners were asked to fill out Quality of Life/Pain questionnaires prior to study, after 48 hours of valproic acid therapy, and at 7 and 21 days following doxorubicin administration. The valproic acid/doxorubicin combination was continued on an every-3-week basis until disease progression or maximal cumulative doxorubicin dose (5 cycles or 150 mg/m²) was reached. Removal from the study resulted from either disease progression, decreased quality of life (VCOG score ≥2), cumulative doxorubicin dose ≥150 mg/m², or owner request. Tumor response in dogs with osteosarcoma that had amputation done was assessed by radiographic evaluation of the size and number of lung metastases.

#### Valproic acid analysis in serum

Serum trough valproic acid concentrations were determined using the Cedia Valproic Acid II Assay (Microgenics) on a Hitachi 917 System Analyzer (Roche). Results were graphed as serum trough valproic acid concentration versus maintenance dose.

#### Doxorubicin and doxorubicinol analysis in plasma by liquid chromatography/tandem mass spectrometry

Doxorubicin and doxorubicinol were measure in dog plasma using a liquid chromatography/tandem mass spectrometry assay. Positive ion electrospary ionization mass spectra were obtained with a MDS Sciex 3200 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Inc.) with a turbo ionspray source interfaced to an Agilent 1200 Series Binary Pump SL HPLC system. Samples were chromatographed with a Phenomenex Prodigy, 5 μm, C18 100 Å, 150 × 2.00 mm column (Phenomenex). A liquid chromatography gradient was employed with mobile phase A consisting of 10 mmol/L ammonium acetate plus 0.1% acetic acid (pH 4.4) and mobile phase B consisting of acetonitrile. Chromatographic separation was achieved holding mobile phase B steady at 10% from 0 to 2 minutes, increasing mobile phase B linearly from 10% to 90% from 2 to 9 minutes, holding steady from 9 to 11 minutes, and decreasing linearly from 90% to 10% from 11 to 13 minutes, followed by re-equilibration from 13 to 15 minutes. The sample injection volume was 50 μL and the analysis run time was 15 minutes. The mass spectrometer settings were optimized as follows: turbo ionspray temperature, 350°C; ion spray voltage, 5,500 V; declustering potential, 20 V; entrance potential, 5.0 V; collision energy, 40 V; collision cell exit potential, 5.0 V; curtain gas, N₂, 50 units; collision gas, N₂, medium. Samples were...
quantified by internal standard reference method in the multiple reaction monitoring mode monitoring ion transitions m/z 544→361 amu for doxorubicin, m/z 546→363 amu for doxorubicinol, and m/z 528→321 for the internal standard, daunorubicin. Scan times were 200 ms, and Q1 and Q3 were both operated in unit resolution mode.

Analytical standards (2.5-500 ng/mL), quality control, and unknowns were all prepared by adding 500 μL of unknown or spiked blank plasma samples to 1.5 mL microcentrifuge tubes containing 5 μL of 10 μmol/L daunorubicin solution followed by brief vortexing. Plasma proteins were then precipitated by the addition of 500 μL of acetonitrile followed by vortex mixing for 10 minutes. Samples were centrifuged at 18,000 relative centrifugal force for 10 minutes, and the supernatant was collected and transferred to autosampler vials for analysis. The lower limit of quantitation for the assay was 2.5 ng/mL for both doxorubicin and doxorubicinol with accuracy and precision (CV%) of 91.7% ± 5.7% and 95.3% ± 3.8%, respectively.

Immunohistochemistry

Deparaffinized sections of pre- and post–valproic acid tumor biopsies were stained for histone H3 acetylation after antigen retrieval using DakoCytomation Target Retrieval Solution pH 9 (Dako). Prepared sections were incubated with anti-AcH3 at 1:50 overnight at 4°C followed by goat anti-rabbit HRP at 1:250 for 1.5 hours at room temperature followed by diaminobenzidine (Vector Labs) staining and hematoxylin counterstain. Images were obtained using a Zeiss Axioplan 2 microscope coupled with a Zeiss AxioCam HRc camera. For blinded comparison of acetylated histones between pre- and post–valproic acid samples, an overall H-score was given to each image, obtained by multiplying the percent of cells within each field with positive nuclear staining (0-100; determined subjectively) by an overall stain intensity score (0 for no evident staining to 3 for intense staining). Seven images were obtained for each pre- and post-treatment sample and H-score results were averaged.

Immunofluorescence

Isolated PBMC from pre- and post–valproic acid blood samples were stained for acetylated histones H3 using total histone H3 as a control. PBMC were isolated using lymphocyte separation media (Mediatech, Inc.). Briefly, two volumes of PBS with 5mmol/L EDTA were added to heparinized blood samples, which were then underlaid with 8 mL of lymphocyte separation media. Samples were spun at 400 × g for 20 minutes and the lymphocyte layer was aspirated and washed three times with one volume of PBS/EDTA. Isolated PBMC were stored in liquid nitrogen until evaluation was done. PBMC were cytocentrifuged onto slides and fixed in 95% ethanol/5% glacial acetic acid at −20°C for 5 minutes prior to permeabilization using 0.2% Tween 20 in PBS. Nonspecific binding was blocked with 1% bovine serum albumin in PBS-Tween 20 for 1 hour at room temperature, followed by overnight incubation with polyclonal rabbit anti-acetyl histone H3 (1:200) or anti-total H3 (1:50) then 1.5 hours with goat anti-rabbit-FITC (1:250). Slides were then rinsed three times and mounted using VectaShield plus 4’, 6-diamidino-2-phenylindole (Vector Labs). Images were obtained using a Zeiss Axioplan 2 microscope coupled with a Zeix AxioCam HRc camera. For blinded comparison of acetylated histones between samples, a semiautomated computerized image analysis program was developed using AxioVision Rel. 4.5 software (Zeiss) that measured the fluorescence of each cell within a field and reported the average fluorescence for each field. Seven images were obtained from each sample and the results were averaged.

Western analysis

For evaluation of tumor tissue histone acetylation, snap-frozen pre- and post–valproic acid biopsy samples were lysed in buffer containing T-PER Protein extraction reagent (Pierce), 1 mmol/L NaVO₄, 1 mmol/L phenylmethylsulfonyl fluoride, Complete Mini protease inhibitor (Roche), and 1% SDS, transferred to 1.5-mL microfuge tubes and

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**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n = 21) No. (%)</th>
</tr>
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<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11 (52.4)</td>
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<tr>
<td>Female</td>
<td>10 (47.6)</td>
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<tr>
<td><strong>Age, years</strong></td>
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<tr>
<td>Median</td>
<td>9</td>
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<tr>
<td>Range</td>
<td>6.2-13.4</td>
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<tr>
<td><strong>Weight, kg</strong></td>
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<tr>
<td>Median</td>
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<td>Range</td>
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<td><strong>Breed</strong></td>
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<tr>
<td>Purebred</td>
<td>16 (76.2)</td>
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<tr>
<td>Mixed</td>
<td>5 (23.8)</td>
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<tr>
<td><strong>Tumor histology</strong></td>
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</tr>
<tr>
<td>Lymphoma</td>
<td>5 (23.8)</td>
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<tr>
<td>Melanoma</td>
<td>4 (19)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>3 (14.3)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>3 (14.3)</td>
</tr>
<tr>
<td>Soft tissue sarcoma</td>
<td>3 (14.3)</td>
</tr>
<tr>
<td>Hemangiosarcoma</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>Mast cell</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>Cutaneous T-cell lymphoma</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td><strong>Dose cohort, mg/kg (loading/maintenance)</strong></td>
<td></td>
</tr>
<tr>
<td>30/15</td>
<td>2 (9.5)</td>
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<tr>
<td>60/30</td>
<td>1 (4.7)</td>
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<tr>
<td>150/75</td>
<td>1 (4.7)</td>
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<tr>
<td>180/90</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>210/105</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>240/120</td>
<td>3 (14.3)</td>
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</table>
passed through a 25-gauge needle 7 to 10 times before centrifugation at 10,000 × g for 10 minutes. Protein concentration of lysates was determined via bicinchoninic acid assay (Pierce). Lysates were loaded into a denaturing 4% to 12% Bis-Tris gel (Invitrogen) and electrotransferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% non-fat dry milk in TBS-Tween 20 [40 mmol/L Tris (pH 7.6), 300 mmol/L NaCl, and 0.5% Tween-20] for 1 hour at room temperature and incubated in a 1:2,500 dilution of rabbit polyclonal anti-acetyl H3, or total H3, in blocking solution overnight at 4°C. After three washes in TBST, membranes were incubated in a 1:20,000 dilution of HRP-conjugated goat anti-rabbit IgG for 1.5 hours at room temperature. Immunoreactive proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and analyzed by autoradiography.

Densitometry was done using Image J software available online from the NIH (http://rsb.info.nih.gov/ij/index.html).

Statistical analyses
Determination of correlations between serum trough valproic acid levels and dose, WBC parameters and valproic acid dose, and fold induction of PBMC histone hyperacetylation versus dose were done using linear regression. Doxorubicin pharmacokinetic parameters were calculated by non-compartmental analysis as previously described (29), and comparisons between our data and historical controls were made by unpaired two-tailed T-test. Comparison of H-scores between pretreatment and posttreatment samples was done by two-tailed T-test. Statistical analysis was done using GraphPad Prism (GraphPad Software). For all comparisons, P < 0.05 was considered significant.

Results
Dose-escalation trial
An accelerated dose-escalation design was used to govern dose escalation toward a maximum tolerated dose.
(MTD) for oral administration of valproic acid. In all, 21 dogs met the inclusion criteria and were enrolled in the study. All dogs underwent pretreatment evaluation of blood chemistry and CBC, and pretreatment biopsies were obtained from accessible tumors. Age, weight, sex, breed, and tumor type were recorded for each patient (Table 1). At enrollment, 10 of 21 patients (47%) had documented metastatic disease, and 8 of 21 (38%) had received prior chemotherapy and/or radiation or had been enrolled in a previous clinical trial.

Oral valproic acid was well tolerated in all dose-escalation cohorts, with only one grade 3 metabolic event reported [alkaline phosphatase (ALKP) > 5× upper limit of normal (ULN)] in the 180/90 mg/kg cohort in a patient with stage V lymphoma. In addition, one grade 2 anorexia was reported in a patient in the 210/105 mg/kg cohort. Other owner-documented side effects were reported as mild, and included lethargy, decreased appetite, and diarrhea. A total of 69 treatment cycles were administered with an average of 3.3 cycles per patient (range, 1-5). No MTD was reached, as the highest dose failed to produce any dose-limiting toxicity. Escalation was halted due to compliance issues with the number of tablets required; owners were required to administer as many as 19 tablets for loading doses followed by up to 9 tablets twice per day thereafter.

Two dogs required dose reductions in doxorubicin after the first treatment cycle because of neutropenia; one patient at the 60 mg/kg maintenance dose exhibited febrile grade 3 neutropenia (neutrophil count 500-999/μL) and another at the 105 mg/kg maintenance dose with nonfebrile grade 3 neutropenia.

Responses were evaluated by Response Evaluation Criteria in Solid Tumors (RECIST; ref. 30) and included 2 of 21 (10%) complete responses (both lymphoma), 3 of 21 (14%) partial responses (both lymphoma, melanoma, lung carcinoma), 5 of 21 (24%) dogs with stable disease through five treatment cycles (osteosarcoma, renal cell carcinoma, apocrine gland adenocarcinoma, melanoma, soft-tissue sarcoma), and 11 of 21 (58%) progressive disease.

Pharmacokinetics

For determination of serum trough valproic acid concentrations, blood was obtained 48 hours after initiation of therapy, immediately prior to administration of the 5th dose. A linear correlation between the administered maintenance dose and serum trough valproic acid concentration existed in the patient population (Fig. 1). In addition, we evaluated doxorubicin pharmacokinetic parameters including area under the concentration-time curve (AUC), clearance, and half-life in three dogs in the highest dose cohort. To determine if administration of valproic acid altered doxorubicin pharmacokinetics, our pharmacokinetic results were compared with those of two previously reported studies evaluating doxorubicin pharmacokinetics in dogs receiving single-agent therapy (31, 32). As shown in Fig. 2, there were no significant
differences in any of the evaluated pharmacokinetic parameters between our study dogs and historical controls.

Pharmacodynamics
To determine if valproic acid therapy was associated with any myelosuppression, or potentiated doxorubicin-induced myelosuppression, we evaluated patient CBC prior to and 48 hours after initiation of valproic acid therapy as well as 7 days after doxorubicin administration. By examining the ratio of total WBC as well as neutrophil counts from day 3 to day 1, we observed no correlation between dose administered and myelosuppression (Fig. 3A). The WBC nadir following doxorubicin is typically around day 7 postadministration, and a comparison of nadir WBC and neutrophil count with valproic acid dose revealed no potentiation of doxorubicin-induced myelosuppression (Fig. 3B and C). Correlations between absolute neutrophil count and administered dose were also evaluated and results were similar to those observed for total WBC (Fig. 3). Taken together, these data indicate that valproic acid administered at doses of up to 240 mg/kg/day does not induce any significant myelosuppression or potentiate doxorubicin-induced myelosuppression.

Immunofluorescence
As described in Materials and Methods, blood was collected prior to and 48 hours after administration of valproic acid for separation of PBMC and evaluation of histone hyperacetylation. Fourteen matched samples were available for comparison of pre- and post-valproic acid histone acetylation by fluorescence immunocytochemistry. Tumor types represented in dogs providing PMBC for analysis included adenocarcinoma, lymphoma, osteosarcoma, soft tissue sarcoma, renal cell carcinoma, melanoma, hemangiosarcoma, cutaneous T-cell lymphoma, and pulmonary carcinoma. The results were recorded as fold change induction of acetylated histone H3, with total histone or total histone H3 as a control. As shown in Fig. 4, there was a significant correlation between the fold induction of acetyl H3 and the administered valproic acid maintenance dose, suggesting that histone hyperacetylation is useful as a pharmacodynamic marker to evaluate 48-hour valproic acid exposure in future clinical trials.

Immunohistochemistry and Western blot
Nine dogs had tumors that were amenable to biopsy pre-and post-valproic acid treatment. For eight of these dogs, formalin-fixed paraffin-embedded samples were evaluated for induction of histone hyperacetylation by immunohistochemistry, whereas snap-frozen biopsy samples were used for detection of target modulation by Western blot. As shown in Fig. 5, histone hyperacetylation in tumor tissues could be detected by immunohistochemistry, and Western blot confirmed similar induction in these same dogs (data not shown). There was no direct correlation between administered valproic acid dose and magnitude of tumor histone acetylation by immunohistochemistry, nor was there a positive correlation between tumor and PBMC histone hyperacetylation (data not shown).

Discussion
Here we report the results of a phase 1 clinical trial evaluating the use of combined valproic acid/doxorubicin therapy in dogs with spontaneously occurring tumors, aimed at defining a safe and biologically effective dose of valproic acid to use in future efficacy trials, as well as validating a toolbox of pharmacodynamic assays that can be applied to future studies of novel HDACi. The dosing scheme of a 48-hour pretreatment period with a HDACi...
prior to doxorubicin was based upon previous findings by our own group as well as others of superior *in vitro* chemosensitization when compared with coadministration in a number of cell types, as well as *in vivo* activity in a canine osteosarcoma xenograft model (6, 22, 33). Additional benefits of pulse-dosing of valproic acid compared with continuous administration include the ability to give higher doses because of the washout period between treatment cycles, as well as ease of compliance and reduced patient cost.

Eight dose cohorts, ranging from 30 mg/kg/day up to 240 mg/kg/day, were evaluated. This study failed to determine a MTD as the highest dose was well tolerated by patients. Consistent with phase I reports of valproic acid as well as other HDACi in humans, the toxicities encountered were generally mild (8, 14, 34–36). The most commonly reported adverse events were grade 1 anorexia and lethargy and grade 1 diarrhea. One grade 3 metabolic toxicity (increase in serum ALP > 5× ULN) was reported in a patient with stage V lymphoma in the 180 mg/kg/day cohort and was most likely reflective of disease progression during the 48-hour valproic acid pretreatment time period. This was only observed in the first treatment cycle for this patient, with ALP levels returning to near normal after the first cycle and remaining stable for the remainder of the study. In addition, one grade 2 anorexia was reported in a dog in the 210 mg/kg/day cohort but no further toxicity was reported after expansion of that cohort. The number of dogs requiring doxorubicin dose reduction in our study did not exceed that expected for dogs receiving doxorubicin alone (37). Although no MTD was reached as there was no reported dose-limiting toxicity in the highest dose cohort, the number of tablets required and owner compliance became a limiting factor in further dose escalation. It has been suggested, however, that the traditional MTD dosing of anticancer agents may not be optimal when using targeted agents, as the determination of biologically effective dose is a more relevant end point and these agents may have the most efficacy with doses below MTD (38–41). It is interesting to note that human phase I studies have identified a much lower dose of 140 mg/kg/day as the MTD for valproic acid (14), whereas we observed no dose-limiting toxicities in dogs at 240 mg/kg/day. This is likely due to pharmacokinetic differences (i.e., volume of distribution or bioavailability) between species as 48-hour trough serum concentrations in human patients seem to be much higher than those seen in dogs given comparable doses on a mg/kg basis. This may also be explained by interspecies allometric scaling parameters that would suggest dogs require higher administered doses to achieve the same drug exposure. Our data may also suggest, however, that dogs require lower overall valproic acid exposure for biological efficacy as, in spite of lower serum valproic acid concentrations compared with human patients, objective responses were observed in our phase I study with a few responses observed in traditionally anthracycline-resistant tumors. It is possible that higher doses could be administered to dogs that would result in plasma concentrations approximating those seen in human patients, and novel oral formulations of valproic acid that allow higher doses to be administered with fewer tablet numbers could help to further elucidate the relationships among dose, exposure, and response in dogs. However, trough serum levels of up to 0.5 mmol/L were reached in this study; a level that was shown to be capable of chemosensitization *in vitro* in our previous work (22). We did not observe any obvious cardiotoxicity in any dogs, although

Fig. 5. Accessible tumors were biopsied before and 48 hours after initiation of valproic acid. Left, photomicrograph of immunohistochemistry for acetylated histone H3 in a dog with cutaneous T-cell lymphoma pre- and post-valproic acid (210/105 mg/kg cohort) showing significant induction of hyperacetylation. Right, graphical representation of immunohistochemical comparisons for all evaluable biopsy samples. Objective responses were measured in dogs 3, 4, and 5. *, significant increase in histone H3 acetylation (P < 0.05).
this was not directly evaluated in this study and the total cumulative doxorubicin dose was low (<150 mg/m²).

Serum trough valproic acid concentrations increased linearly with the administered maintenance dose. The relative weakness of this correlation could be explained by discrepancies in the times that valproic acid was actually administered to dogs and those reported by owners, or by reduced drug exposure due to postadministration emesis and/or passage of partially digested pills as was reported by a few owners. Pharmacodynamic evaluation of fold change in histone H3 acetylation in 14 evaluable PBMC samples also correlated with administered dose, but did not correlate with trough valproic acid levels (data not shown). This would suggest that the use of a pharmacodynamic end point, in this case direct target modulation, may give a better estimate of overall drug exposure and activity during the 48-hour treatment period than determining valproic acid concentrations at a single time point. In this study, PBMC acetylation was not a predictor of objective response. In addition, tumor histotype did not seem to correlate with degree of target modulation in PBMC. However, these assessments may be limited by the small sample size in this study.

We measured doxorubicin pharmacokinetics in three dogs to ensure that valproic acid pretreatment had no effects on doxorubicin elimination. Because valproic acid pharmacokinetics were linear in our dog population, it would be safe to assume that any changes to doxorubicin pharmacokinetics would be manifest in the highest dose cohort, and these three dogs were used for doxorubicin pharmacokinetic comparisons. We did not anticipate any alterations in doxorubicin pharmacokinetics as the mechanisms of metabolism are nonoverlapping; valproic acid is primarily metabolized by complete β-oxidation, whereas doxorubicin metabolism occurs primarily via reduction by aldo-keto reductase to form doxorubicinol (31, 42). There were no significant changes in the pharmacokinetics of doxorubicin or the major metabolite doxorubicinol when compared with two separate historical control populations receiving doxorubicin alone, suggesting that no alterations in doxorubicin dosing are required in dogs receiving combination therapy. Our results of a lack of potentiation of anthracycline-induced side effects are consistent with a report in humans evaluating a combination of valproic acid and epirubicin, although this report did not specifically evaluate AUC, clearance, or half-life of epirubicin in valproic acid–pretreated patients (14).

Treatment responses were evaluated by RECIST criteria. Two complete responses were observed, both in dogs with lymphoma. Responses in lymphoma with doxorubicin alone are not unexpected as reported remission rates for treatment-naïve lymphoma in dogs range from 60% to 85% (43). For this reason, the number of lymphoma dogs in the study was limited to five. Three dogs experienced a partial remission and included one lymphoma, one melanoma, and one carcinoma. In five dogs, stable disease persisted until treatment was stopped after reaching the maximum doxorubicin dose as opposed to disease progression. Eleven dogs were removed from the study because of progressive disease. No dogs were removed from study due to decreased quality of life (VCOG > 2) or owner request. Although a portion of the objective responses measured may be attributable to doxorubicin alone, it is interesting to note that two of the responses (one partial remission and one stable disease) were seen in melanoma, a tumor that is generally resistant to anthracycline therapy in dogs (44).

In addition to showing target modulation in PBMC following valproic acid treatment, we also found histone H3 hyperacetylation of tumor samples in 4 of 8 (50%) evaluable samples by immunohistochemistry, and Western blot of these samples confirmed histone hyperacetylation. Tumor tissue target modulation did not seem to predict those dogs with a measurable objective response; however, intrinsic doxorubicin sensitivity of each tumor type may overshadow this type of prediction in this combination study. There was no apparent correlation between administered dose and target modulation in tumor tissue, supporting the idea that dosing to toxicity does not increase likelihood of enhanced therapeutic effect. The lack of correlation between dose and tumor target modulation could also be explained by sampling error; obtaining small biopsies from large heterogeneous tumors may result in posttreatment sampling of areas with different basal acetylation levels or varying blood flow resulting in differences in drug exposure.

In conclusion, this is the first study to evaluate the safety and clinical utility of HDACi in dogs with spontaneous cancer, an extremely useful model for bridging the gap between mouse and human clinical studies. We used a sustained-release formulation of valproic acid given for 48 hours prior to a standard dose of doxorubicin and showed that serum trough valproic acid level increased linearly with the dose administered. In addition, we found no evidence that the administration of valproic acid altered AUC, half-life, or clearance of doxorubicin. Valproic acid administration did not result in significant myelosuppression nor did it potentiate doxorubicin-induced myelosuppression at valproic acid doses up to 240 mg/kg/day. We were able to show target modulation, specifically histone H3 hyperacetylation, in both normal and tumor tissues after administration of valproic acid, and the magnitude of PBMC hyperacetylation correlated positively with the administered dose of valproic acid. Interestingly, serum trough valproic acid concentrations did not correlate with magnitude of histone hyperacetylation, suggesting that this particular pharmacodynamic marker may be a better overall determinant of 48-hour valproic acid exposure than trough valproic acid concentrations. Objective responses were observed in this phase I study, and it is of interest to note that a few responses were seen in traditionally anthracycline-resistant tumors. In this study, we have shown the safety of HDACi in dogs with cancer and have developed the tools necessary for rigorous pharmacokinetic/
pharmacodynamic evaluation in future trials of valproic acid or other HDACi.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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