Targeting MEK in a translational model of histiocytic sarcoma

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Abbreviations list:
BMD: Bernese mountain dog
ECD: Erdheim-Chester disease
H&E: hematoxylin and eosin
HHS: hemophagocytic histiocytic sarcoma
HS: histiocytic sarcoma
HTS: high throughput screening
IACUC: institutional animal care and use committee
IHC: immunohistochemistry
LCH: Langerhans cell histiocytosis

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Competing interests
The authors declare that they have no competing interests.
Abstract

Histiocytic sarcoma in humans is an aggressive orphan disease with a poor prognosis as treatment options are limited. Dogs are the only species that spontaneously develops histiocytic sarcoma with an appreciable frequency, and may have value as a translational model system. In the current study, high throughput drug screening utilizing histiocytic sarcoma cells isolated from canine neoplasms identified these cells as particularly sensitive to a MEK inhibitor, trametinib. One of the canine cell lines carries a mutation in PTPN11 (E76K), and another one in KRAS (Q61H), which are associated with the activation of oncogenic MAPK signaling. Both mutations were previously reported in human histiocytic sarcoma. Trametinib inhibited sensitive cell lines by promoting cell apoptosis, indicated by a significant increase in caspase 3/7. Furthermore, in vitro findings were successfully re-capitulated in an intrasplenic orthotopic xenograft mouse model which represents a disseminated aggressive form of histiocytic sarcoma. Mice with histiocytic sarcoma xenograft neoplasms that were treated with trametinib had significantly longer survival times. Target engagement was validated as activity of ERK, downstream of MEK, was significantly downregulated in neoplasms of treated mice. Additionally, trametinib was found in plasma and neoplastic tissues within projected therapeutic levels. These findings demonstrate that in dogs, histiocytic sarcoma may be associated with a dysfunctional MAPK pathway, at least in some cases, and may be effectively targeted through MEK inhibition. Clinical trials to test safety and efficacy of trametinib in dogs with histiocytic sarcoma are warranted, and may provide valuable translational information to similar diseases in humans.
Introduction

Histiocytic and dendritic cell neoplasms encompass a group of proliferative entities with variable clinical behaviors and prognosis in humans (1). Among them, histiocytic sarcoma (HS) is by far the most aggressive disorder with the worst prognosis (2). It is a rare hematological malignancy (< 1% of all hematopoietic neoplasms) (3, 4) that affects all ages, but mostly adults, with a male predominance (5). Existing data show that HS develops within lymph nodes and at extranodal sites including the gastrointestinal tract, spleen, lungs, and head and neck (2, 5, 6). Given the limited occurrence, there is no consensus on standard-of-care treatment for HS in human patients. While surgical resection and radiation therapy are attempted for local control, metastases frequently occur, and in these cases, systemic chemotherapy with regimens of drugs used for lymphoma is often utilized (6-8). More options of medical intervention are needed to improve patients’ survival time, which does not extend beyond one year (9, 10). Although the etiology of HS is not yet clear, there is evidence suggesting a central role of the oncogenic RAS-RAF-MEK-ERK signaling pathway (11-15). A number of HS cases in humans were reported to carry activating mutations in BRAF (V600E, F595L) (11-14, 16, 17), KRAS (Q61H) (15) and HRAS (Q61R) (14), with some cases showing favorable response after targeted therapy with vemurafenib, a BRAF inhibitor, and with MEK inhibitors, including trametinib (12, 16, 18). Other histiocytic and dendritic cell neoplasms including Langerhans cell histiocytosis (LCH) and Erdheim-Chester disease (ECD) seem to share the MAPK oncogenic pathway based on recurring activating mutations in BRAF (V600E, N486_P490del), MAP2K1, and ALK gene fusion (19-23).

Similar to humans, dogs also present with spontaneously occurring forms of histiocytic disorders, with HS being the most aggressive entity. Clinical presentation and biological behavior are variable across these disorders, ranging from benign and self-resolving (i.e. cutaneous histiocytoma), inflammatory (i.e. reactive histiocytosis), to highly aggressive entities (hemophagocytic HS). HS is a rare disease in dogs (<1% of all cancers) (24), however, it is overrepresented in certain breeds, especially in Bernese
mountain dogs (BMD) (incidence of about ~25% in the population) (25). Other breeds that also present with HS include flat-coated retrievers, golden retrievers and Rottweilers. Males and females are equally affected, and dogs are most often in adult age (8-10 years) (26, 27). In dogs, HS presents most commonly in the skin, bone/joint, spleen, lymph node, lungs, and liver (27-29). Due to a high incidence of metastasis, reported as 70-90% (27, 28, 30-32), systemic chemotherapy is the treatment of choice with drugs such as lomustine and/or doxorubicin, resulting in a median survival time of 3-6 months (27, 31, 33, 34). Although the disease etiology is unclear, the deregulation of the tumor suppressor genes MTAP/CDKN2A/B located within the region homologous to human chromosome 9p21 (35, 36) has been implicated in genome wide association studies. As in humans, the MAPK pathway seems to contribute to HS in dogs, as exemplified through a gain-of-function mutation PTPN11<sup>E76K</sup>, a gene that encodes SHP2, required for MAPK. This mutation was recently reported in canine HS by our group, and found to be prevalent in BMDs compared to other breeds (37% vs. 9%) (37).

The immunohistochemical profile is critical to differentiate histiocytic diseases in both humans and dogs, which often present a high level of pleomorphism. Various cell markers are used to characterize cells by their subtype and activated state (Supplementary Table S1). In humans, CD68, lysozyme and CD163 are markers designated for use for HS, with the latter considered of highest specificity (38-40). The diagnosis in dogs is based on different cell markers, CD18 (25, 28, 29, 41), a leukocyte integrin, and CD204, a macrophage scavenger receptor (42, 43). Thongtharb et al. reported a strong positive expression of CD163 in 17 out of 23 cases of canine HS, suggesting that CD163 might be a common marker between the two species (44).

As the only species that spontaneously develops HS, dogs may have value as a translational model for this rare disease in humans. To date, there is no human HS cell line available for study. We have derived and characterized canine HS cell lines, one of which has been described in detail (45). We were able to capitalize on this opportunity by using HS cell lines established from dogs as tools for
the systematic discovery of novel treatment approaches. A high throughput screening (HTS) of about 2,000 compounds identified trametinib, a MEK inhibitor, as a highly potent drug against canine HS cells. Trametinib is an FDA-approved drug for patients with advanced melanoma carrying a BRAF V600E or V600K mutation (46). Other activating mutations in NRAS, KRAS and HRAS, upstream of BRAF, are also susceptible to MEK inhibition (47, 48). Here, we report the use of trametinib as an effective inhibitor against three canine HS cell lines, two carrying known activating mutations previously reported in HS human patients (15, 18, 48), one carrying the PTPN11<sup>E76K</sup>, and another KRAS<sup>Q61H</sup>, reported for the first time in dogs in this study. We demonstrate that the inhibition of MEK promotes cell cycle blockage with arrest of sensitive HS cell lines in G1 phase, and that most specifically, MEK inhibition triggers a significant increase in apoptosis. This effect seen <em>in vitro</em> with trametinib was then recapitulated in mice harboring HS xenografts, where we demonstrate intratumoral target engagement. Our study indicates that trametinib may be a promising targeted therapy for canine HS, and provides a rational for the initiation of clinical trials and further understanding on safety and anti-tumor efficacy in dogs, especially when the tumors carry activating mutations in MEK pathways. Importantly, we expect that our findings can be potentially translated to similar human diseases driven by a deregulated MAPK signaling pathway.

**Material and Methods**

**Cell culture establishment and maintenance**

Neoplastic tissues were collected from client owned dogs at post-mortem examination, with owner’s written consent, approved as an “exempt” procedure by MSU Institutional Animal Care and Use Committee (IACUC). Detailed characteristics of tissue donors are listed in <strong>Supplementary Table S2</strong>. Minced tissues were disaggregated in 1% collagenase (C9891, Sigma) for one hour, then transferred to medium with 10% heat-inactivated fetal bovine serum, 1% antibiotic-antimycotic 100X, and 0.1% gentamycin (ThermoFisher), and incubated at 37°C in 5% CO<sub>2</sub>. For medium, RPMI1640
(ThermoFisher) was used for BD, OD and PJ, whereas DMEM (ThermoFisher) was used for fibroblasts. DH82, a commercially available cell line derived from a macrophage derived sarcoma, hemophagocytic HS (HHS) (CRL-10389, ATCC), was kept in EMEM (ATCC).

**Fingerprinting of cell lines**

Genomic DNA from HS cell lines BD, PJ, and OD was submitted for analysis of genetic profiles (CellCheck Plus, IDEXX). All HS cell lines were confirmed to be of canine origin with no mammalian interspecies contamination. Based on a panel of STR markers, there was no cross contamination across cell lines, and their individual genotypes were identical to samples from early cell passages or whole blood from the original donor. A genetic profile of microsatellite markers is available in **Supplementary Table S2** and can be used for future monitoring.

**Characterization of cell lines**

For immunohistochemistry (IHC) analysis of the cell lines BD, OD, PJ, DH82 and normal fibroblasts, cell pellets were first embedded into histogel for stability, then fixed in 10% formalin for up to 17hs and transferred to 70% ethanol until embedding in paraffin. Pellets were embedded into paraffin following routine methods. Sections from the cell pellets were rehydrated and labeled with monoclonal antibodies against CD3, CD18, CD79a and CD204, following standard procedures at the MSU Veterinary Diagnostic Laboratory. Images were acquired using a Nikon H600L Microscope as brightfield images using a 10X dry objective. The images were analyzed using NIS-Elements AR 3.1 software.

In order to evaluate for the presence of mutations in the genes BRAF/NRAS/KRAS/HRAS, sequences of the coding regions were determined from RNA-seq data from the HS cell lines. For this purpose, messenger RNA was isolated, assayed for quality and sequenced using the Illumina HiSeq 4000 platform to generate a minimum of 150 million reads at 2X150 BP at the MSU Genomics Core. Data acquired from sequencing was checked for quality using FastQC and low quality bases were trimmed using TrimGalore.
High throughput screening

Cell lines BD (8,500/well) and DH82 (6,500/well), and normal fibroblasts (4,000/well) isolated from skin were plated into 384-well plates. A collection of 1,952 compounds from Prestwick (Prestwick Chemical), Approved Oncology Drugs Set V (NCI Developmental Therapeutics Program) and Published Kinase Inhibitor Set (PKIS, Structural Genomics Center, UNC) libraries were delivered to cells using a Biomek FX Workstation liquid handling system (Beckman Coulter) at a single concentration of 1 μmol/L for 48hs. CellTiter-Glo Cell Viability (G7570, Promega) reagent was added and the luminescence signal was read using a Synergy Neo (BioTek) detection platform. Drugs were selected for further analysis based on the following cutoffs: >20% inhibition in one or both HS cell lines, and <20% inhibition in control normal fibroblasts. Compounds of unknown mechanisms of action were excluded.

The OD and PJ cell lines were not yet established at the time of the HTS studies, but were included in further assays, as they became available.

Dose response confirmation assay

Selected compounds from HTS were re-tested in a dose response assay, so their pIC50 values could be used to determine their cytotoxic potency. Cell lines BD (8,500/well), DH82 (6,000/well) and PJ (8,500/well), and normal fibroblasts (8,500/well) were seeded into 384-well plates. The dose response assay was performed prior to the establishment of OD cell line, not included in this assay. Selected compounds were added in 8 escalating concentrations for 48hs. Cell viability was measured as explained above for HTS. pIC50 of each compound was calculated by GRETL software within MScreen, a HTS data storage and analysis system (Center for Chemical Genomics, University of Michigan)(49).

Cell viability assay

HS cell lines BD (3x10⁴/well), DH82 (1.5x10⁴/well), PJ (3x10⁴/well) and OD (3x10⁴/well), and normal fibroblasts (2x10⁴/well) seeded on 96-well plates were treated with Trametinib (16292, Cayman) in
1%DMSO for 72hs. Viability of cells was determined using CellTiter 96® Aqueous Proliferation Assay (G3581, Promega). The formazan product was measured using an EnVision plate reader (PerkinElmer). Results were plotted using GraphPad Prism 6 software nonlinear regression curve fitting (GraphPad Software) to calculate the pIC50 of each compound.

**Cell cycle assay**

HS cell lines BD, OD, PJ and DH82, and normal fibroblasts were treated with 1nM, 10nM, 100nM and 1µM of trametinib for 24hs. Cells were fixed in 70% ethanol for >3hs at 4°C, DNA was stained with 50µg/ml propidium iodine, and RNA cleaned out with 16µg/ml RNase A at 4°C. For analysis of DNA content, the flow cytometer BD LSR II (BD Bioscience) was used, and for data interpretation, ModFit LT V4.1.7 software (VSH) with auto fit and auto linearity settings.

**Evaluation of caspase 3/7 activity**

HS cell lines BD, OD, PJ and DH82, and normal fibroblasts plated in a white 96-well plate were treated with 1nM, 10nM, 100nM and 1µM of trametinib for 24hs. Caspase-Glo 3/7 Assay (G8090, Promega) reagent was added to the cells for 30minutes, and luminescent signal was detected using an EnVision plate reader (PerkinElmer). Cells treated with 1%DMSO were used as baseline measurement of apoptosis. As an apoptosis inducer, staurosporine (81590, Cayman) was used as a positive control at 10µM (50, 51).

**Western blot**

HS cell lines BD, OD, PJ, DH82 and normal fibroblasts treated with either vehicle (0.1%DMSO) or trametinib for 2hs were lysed using CellLytic M (C2978, Sigma-Aldrich) supplemented with 1:100 protease inhibitor (P8340, Sigma-Aldrich) and phosphatase inhibitor B (sc-45045, Santa Cruz). Pre-weighed splenic tissues containing xenograft neoplasms were briefly homogenized in CellLytic MT (C3228, Sigma-Aldrich) with 1:100 protease and phosphatase inhibitor using TissueRuptor (Qiagen). Total protein from supernatant was quantified using Qubit (Thermo Fisher). A total of 40-50µg of
protein per lane were separated by Novex NuPAGE SDS-PAGE (10%), and transferred to PVDF membrane. Membranes were blocked with 5%BSA in TBS buffer, and probed overnight with primary antibodies at 4°C (Supplementary Table S3). Secondary antibodies were incubated for 1h at room temperature. Image of bands was detected using Odyssey Imaging System (LI-COR) and analyzed with Image Studio™ Lite software (LI-COR).

Transfection of luciferase vector

Neoplastic cells from the BD cell line were transfected with a luciferase vector to enable cell tracking by In Vivo Imaging System (IVIS). Briefly, BD cells were incubated with vector pGL4.51[luc2/CMV/Neo] (E1320, Promega) and Lipofectamine 2000 (11668027, ThermoFisher) for up to 48hs. Transfected cells were selected by Geneticin (G418, ThermoFisher) at 140μg/ml. A bioluminescent signal was detected in as low as 12,500cells/well in vitro. BD cells transfected with luciferase will be referred as BD-luc.

Orthotopic xenograft mouse model of HS

Immunodeficient 6-week old, female of NOD scid gamma mice (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ, JAX) under general anesthesia with 2-3% isoflurane, were injected aseptically with 50µl of 1.5x10<sup>6</sup> BD-luc cells mixed 1:1 with Matrix Matrigel (CB-40234C, Corning) directly into the spleens, through a laparotomy approach. After 14 days, mice were randomly divided into two groups of five each (treatment group and vehicle control group). Mice were treated daily either with 1mg/kg of trametinib (16292, Cayman) or vehicle β-cyclodextrin 10% (16169, Cayman) intraperitoneally (IP). Animals were euthanized when humane end-points were reached or after 35 days post cells injection. This xenograft study was approved by the MSU IACUC (AUF# 09/15-133-00). Humane endpoints observed were as follows: body condition score <2, weight loss >15%, lack of movement, abnormal posture and haircoat, and/or clinical signs score ≥15 (Supplementary Table S4). Additionally, the
animals’ health was monitored daily by the veterinary staff of the university’s Campus Animal Resources.

**In vivo bioluminescence imaging**

For monitoring neoplastic growth, mice were imaged using the IVIS Spectrum (CaliperLS) instrument twice a week under anesthesia with isoflurane. Mice were injected with D-luciferin (LUCK-100, GoldBio) 150mg/kg IP and images were acquired 15 minutes later. Bioluminescent radiance (p/sec/cm²/sr) from each animal was analyzed using Living Image (Caliper) software.

**Mass spectrometry analysis**

Plasma samples were mixed with acetonitrile in a 1:3 ratio for protein precipitation. A total of 25mg of tissue samples (muscle, heart, brain, kidney, spleen and liver) were briefly homogenized in 100μl PBS buffer using TissueRuptor (Qiagen), and then mixed with 300μl of acetonitrile. Homogenates were centrifuged at 7,500rpm for 10minutes at 4°C, and supernatant collected for analysis. Internal standard (propyl-4-hydrobenzoate) was added to all samples for normalization. Samples were run on the Quattro Premier triple quadrupole LC/MS/MS instrument using an analytical column (53822-U, Ascentis® Express, C18, 5cmx2.1mm, 2.7μm, Supelco, Bellefonte) at 0.4ml/min. Peak integration was done using Quanlynx software (Masslynx).

**Histopathology of xenograft mouse tissues**

Tissues from each mouse were fixed in 10% formalin for 24hs and transferred to 70% ethanol until embedding in paraffin. Tissues collected included spleen, liver, heart, lung, brain, intestine, pancreas, stomach, kidney, adrenal gland, skeletal muscle, ovary, and uterus. For each mouse, one representative section of each organ was stained with hematoxylin and eosin (H&E) stain. For IHC, sections from the paraffin blocks were deparaffinized in xylene and rehydrated in ethanol at different concentrations. Hydrogen peroxide 3% was used to neutralize endogenous peroxidase. Antigen retrieval was performed on the PT Link (Dako NA), using the EnVision FLEX Target Retrieval
Solution, low pH (Dako NA) for 20 minutes. Sections were labeled with a monoclonal antibody against CD204 (KT022, clone SRA-E5, Transgenic), using an Autostainer Link 48 and the EnVision Flex+ detection system (Dako). Immunoreaction was visualized with 3,3′-diaminobenzidine substrate (Dako) and sections were counterstained with haematoxylin. Images were acquired using a Nikon H600L Microscope as brightfield images using a 10X dry objective. The images were analyzed using NIS-Elements AR 3.1 software. Assessment of histopathology of treatment and vehicle groups was performed by a board certified veterinary pathologist (S.C.) who was blinded as to the treatment status of each sample.

Assessment of liver function

Plasma samples collected immediately post-mortem from mice from both treatment and vehicle groups were sent for analysis of albumin, AST and ALT at the In-vivo Animal Core (IVAC, University of Michigan). As a control group, plasma samples were obtained from naïve mice of the same strain which had not been transplanted with tumors. All animals had a similar age, had the same diet and were housed under the same conditions.

Results

Cell lines established from tissues of dogs

Three cell lines were established from HS of dogs. Two cell lines originated from BMD (BD and OD), the breed with the highest incidence of HS, and one from a Rottweiler (PJ). A comprehensive characterization of the BD cell line was recently published by our group (45). To confirm the histiocytic phenotype, we used two markers that are routinely used to diagnose canine HS: CD18 (integrin beta chain beta 2) and CD204 (class A macrophage scavenger receptor) (25, 28, 29, 41, 43). All three cell lines were positive for both markers by IHC, while negative for CD3 and CD79a, ruling out a lymphoid phenotype (Supplementary Figure S1). The BD cell line carries the PTPN11<sup>E76K</sup> mutation.
Coding regions of the genes BRAF/NRAS/KRAS/HRAS from the cell lines were evaluated for the presence of mutations. We identified that the KRAS Q61H mutation is present in OD cell line.

Alignment of regions of KRAS and PTPN11 mutations from HS cell lines and other relevant species can be found in Supplementary Table S2. No additional mutations were identified in any of the cell lines. Gene sequences can be found under Sequence Read Archive (SRA) study accession SRP139948.

Drug screening identifies MEK inhibitor as a drug candidate for HS

Results from HTS indicated trametinib, a MEK inhibitor, as one of the few compounds highly effective against at least one HS cell line (pIC50>7), BD cell line, while safe for normal cells (pIC50<5). To confirm these observations, we performed cell viability assays using four HS cell lines. Three of them were particularly sensitive to trametinib: BD, OD and PJ (Figure 1A). In contrast, the DH82 HHS cell line was resistant to trametinib with at least one order of magnitude lower pIC50 (5.8). Two populations of fibroblasts isolated from skin biopsies/necropsy tissue of dogs were used as normal control, one originated from a mixed breed dog, and one from the dog from which the OD cell line had originated. Both populations of fibroblasts had markedly lower pIC50s, specifically 3.6 and 4.7 vs. 6.7, 7.5 and 7.8 from PJ, BD and OD cell lines, respectively.

Trametinib blocks cell cycle progression in HS cell lines

To verify whether trametinib inhibits HS by disrupting the cell cycle, we performed a propidium iodine based assay. The inhibition of MEK blocked cell cycle progression in all HS cell lines (BD, OD, PJ and DH82) at all ranges of concentrations of trametinib, low and high (Figure 1B). Trametinib promoted a significant increase of HS cells in G1 (p≤0.001), and a subsequent decrease in S and G2 phases of the cell cycle, in comparison to untreated cells. In contrast, cell cycle progression of fibroblasts was not affected by trametinib, as the population of cells in each phase of the cell cycle remained unchanged. In general, fibroblasts had a higher percentage of cells in the G1 phase regardless of treatment although they were in an active proliferative state.
Apoptosis is augmented in HS cell lines sensitive to trametinib

To further understand the mechanisms through which trametinib inhibits HS, we assessed the effect on apoptosis based on caspase 3/7 activity after treatment. Interestingly, the level of apoptosis was significantly increased only in the HS cell lines sensitive to trametinib (BD, OD and PJ) vs. DH82 and normal fibroblasts (p≤0.05) (Figure 1C). Apoptosis was enhanced with treatment at low concentrations, with all sensitive cell lines presenting at least a two-fold increase in apoptosis at 10nM of trametinib. These results demonstrate that the effect of trametinib on induction of apoptosis is a critical distinction between sensitive and resistant HS cell lines.

PI3K-AKT pathway is upregulated in resistant hemophagocytic HS cell line

Using phosphorylated ERK (p-ERK) as a readout for MAPK pathway activation, we confirmed that the pathway was substantially inhibited with trametinib in a dose dependent manner: p-ERK was decreased at 10^{-8}M, and markedly reduced at 10^{-7}M in all cell lines (Figure 2A). We also evaluated key elements upstream of ERK of the MAPK pathway on cells treated with trametinib 10^{-7}M concentration, as this was sufficient to obliterate p-ERK in all cell lines (Figure 2B). Expression of BRAF and RAS was similar in intensity across cell lines, and did not change with trametinib. Since the MAPK pathway crosstalks with the PI3K/AKT pathway in a way that one can counterbalance the inhibition of the other, we evaluated main components of PI3K/AKT signaling. Indeed, the pathway was more activated in the resistant cell lines, DH82 and normal fibroblasts, represented by increase in p-AKT. Interestingly, expression of the tumor suppressor PTEN, an inhibitor of the PI3K-AKT pathway, was nearly undetectable in the DH82 cell line. Expression of p-AKT and PTEN remained unchanged after treatment with trametinib in all other cell lines.

In order to evaluate possible off targets events under treatment with trametinib, we also looked at components of the p38 and JNK pathways. While p-p38 was not affected by the treatment, expression of p-JNK was undetectable in the neoplastic cell lines, and unchanged in fibroblasts treated with trametinib.
Administration of trametinib inhibited tumor growth and prolonged survival time in a xenograft mouse model

To determine whether the sensitivity to trametinib could be re-capitulated in vivo, we used an intrasplenic orthotopic xenograft mouse model with BD-luc cells. Tumor growth correlated with an increase in bioluminescent signal over time as shown in Figure 3A, and graphically represented in Figure 3B. Mice treated with trametinib had a significantly lower HS growth rate in comparison to untreated mice (p=0.02).

Mice were euthanized when the pre-determined humane endpoints were reached. All mice in the control group reached the endpoints prior to 30 days post transplantation, and were humanely euthanized on days 21 (n=2), 23, 25, and 30 post injection of cells, due to their poor health conditions. In contrast, all mice from the treatment group were alive and in fair condition by the end of the study period, at 37 days post injection. Despite the small cohort of mice in each group, mice from the treatment group exhibited a significantly longer survival time (p=0.002), represented in the Kaplan-Meier survival curve (Figure 3C).

MAPK signaling was inhibited in HS of mice treated with trametinib

In order to evaluate if the response seen in the mice was associated with inhibition of the target MAPK pathway, we collected HS neoplasms from spleens of mice 24hs post treatment to determine expression of p-ERK. The MAPK pathway was significantly inhibited in tissues from the treatment group (p≤0.01), represented by a decrease in p-ERK (Figure 4A). In HS tissues (spleen), the concentration of trametinib was consistent across animals with an average of 21.4nM (SD=3.2, range:17-25) (Figure 4B). The drug was also detected in other tissues and in circulation at variable concentrations. Muscle tissues had the lowest concentrations of trametinib, while liver tissues had the highest concentrations. Trametinib was undetectable from tissues and plasma from mice treated with vehicle only.

Trametinib decreased tumor growth in the liver and minimized tumor-associated liver injury
After euthanasia, tissues were immediately harvested from all mice for evaluation of metastases. H&E sections of spleen confirmed that all mice in both treatment and vehicle groups had diffuse infiltration of neoplastic cells. Metastases were present in the liver histologically, confirmed by CD204 IHC to be of tumor origin (Figure 4D), and also in the mesentery and pancreas (Supplementary Figure S2). Percentage of cell necrosis within the splenic and hepatic tissues were scored using a semi quantitative system, as described by Gibson-Corley et al. (52). When compared to treated mice, the vehicle control group had a significantly higher score of necrosis in the liver (p≤0.05) and in the spleen (p≤0.01), using the unpaired t-test (Figure 4E). The mitotic count of the splenic neoplasms was similar between both groups (p=0.916 - unpaired t-test), with a mean (standard deviation) of 42(9.7) vs. 42.7(10.8), and a median of 42 vs. 42.5 mitoses per 10 high-power (400X) fields, for treatment group and control group, respectively.

Furthermore, histologic features of liver injury were more prominent in hepatocytes from vehicle control mice and included hepatocellular coagulative necrosis (5/5 mice), microvesicular vacuolar change (5/5 mice), and cytoplasmic hypertrophy (4/5 mice). In contrast, hepatocytes from treated mice had either minimal microvesicular vacuolar change (1/5 mice) and mild hepatocellular atrophy (1/5 mice), or were histologically normal (3/5 mice).

Liver function tests were conducted from treated and untreated control mice, and untreated naïve non tumor-bearing mice to evaluate the effect of trametinib on hepatic function. Albumin levels of the untreated naïve mice were significantly higher than those mice from control group (p≤0.001) and trametinib group (p≤0.01). Liver enzyme ALT was significantly higher in the vehicle control group vs. the untreated naïve group of mice (p≤0.05) and the trametinib group (p≤0.05) (Figure 4C).

Discussion
Our findings provide further support that the MAPK pathway may represent an important oncogenic driver in canine HS, and provide proof of concept data for initiating clinical trials with trametinib in dogs with HS. While the low incidence of HS in humans constitutes a major challenge to both the study of oncogenesis, as well as the initiation of clinical trials, the dog may represent a unique translational model for this orphan disease. Further molecular characterization on both human and dog HS tumors are needed to fully evaluate the inter- and intra-species variation that may exist in these malignancies.

In the present study, we identified trametinib, a MEK inhibitor, as an effective drug against canine HS at concentrations demonstrated to be achievable in plasma (53, 54). Trametinib is an allosteric, non-ATP-competitive compound with sub-nanomolar activity against MEK1/2, in the MAPK pathway, a major oncogenic driver in multiple malignancies (48). It emerged as a promising treatment for melanomas carrying activating mutant RAF and RAS (46-48), and led to a successful combination therapy, with dabrafenib, a BRAF inhibitor, for a multi-target strategy for melanoma (55) and non-small cell lung cancer (56). It is currently part of several advanced clinical trials for other solid tumors and leukemia (57).

We validated the inhibition of the target MEK by documenting a decreased expression of p-ERK, a downstream readout for this pathway, in the cells treated with trametinib. We also evaluated the presence of off target effects by looking at proteins representing other branches of MAPK network, p38 and JNK, which were unchanged by treatment with trametinib. However, the existence of off targets events cannot be completely ruled out without an investigation at the level of global proteomic activity.

Although the etiology of human HS is unclear, there is evidence suggesting the role of an oncogenic MAPK pathway, based on cases harboring activating mutations in key genes. BRAF (V600E, V595L, G464V, G466R) (11, 12, 14, 15, 21), HRAS (Q61R) (14), KRAS (Q61H) (15), and MAP2K1 (16, 58) which have been reported; however, because of the small number of cases, their incidence in HS is
still unknown. In a few cases, targeted therapy with small inhibitors vemurafenib and trametinib, a 
BRAF and a MEK inhibitor, respectively, was associated with favorable response (12, 18). Among 
those key genes, we detected a mutation in one of the cell lines, \textit{KRAS}^{Q61H}, in the OD cell line. The 
\textit{KRAS}^{Q61H} mutation is known to be an activator of MAPK signaling, and previously reported in a 
human HS by Liu et al. (15). Mutations in KRAS are present in 22% of all cancers (59) with 82.5% of 
those cancers being sensitive to trametinib, as reported by Jing et al. where 33 of 40 (82.5%) KRAS 
mutant cell lines showed IC_{50}<50nM (48, 60). Genes in this pathway are highly conserved. There is a 
high degree of identity at the amino acid level between human and dog sequences with BRAF, 
KRAS, HRAS, and NRAS, showing 99%, 99% 98% and 100% identities, respectively. Therefore, it is 
not surprising to see activating mutations be shared among the two species. No other mutation was 
found in BRAF, KRAS, NRAS and HRAS across all HS cell lines.

As recently reported by our group, a gain of function mutation in PTPN11 was found to be associated 
with HS in BMDs (37). Activating mutations in the PTPN11 gene have been also reported in human 
HS cases (15, 18, 61, 62). PTPN11 encodes the SHP2 protein, which is required for the MAPK 
pathway. Interestingly, among our HS cell lines, BD carries the mutation PTPN11^{E76K}, previously 
reported in two human HS cases (18, 61). In one of these cases, the patient with multi-organ HS 
received trametinib after failing several chemotherapy protocols, resulting in a partial remission for 
two months (18). Therefore, we hypothesize that the sensitivity to trametinib of BD and OD cell lines 
due to the presence of mutations that activate the oncogenic MAPK pathway in these cell lines. We 
could not detect mutations in BRAF/HRAS/KRAS/NRAS on a third sensitive cell line (PJ), suggesting 
that other genes that activate MAPK should be investigated through a more comprehensive mutation 
analysis, currently underway in our group.

Trametinib induces p15^{INK4b} and p27^{KIP1}, inhibitors of CDK4/6 (63), leading to the arrest of the cell 
cycle at the G1 phase (64, 65). Similarly, trametinib significantly blocked cell cycle progression in the 
G1 phase, however, the effect was similar in magnitude in both sensitive and resistant HS cell lines.
In contrast, the level of apoptosis was significantly augmented in sensitive cell lines (BD, OD and PJ) at concentrations close to their IC50s, while minimal in resistant cells (DH82 and FB), indicating that apoptosis is a key event of growth suppression in HS cells by trametinib, and that it may represent a marker for sensitivity to this drug. Likewise, induction of apoptosis has been reported as a major mechanism of inhibition in other cancer cells treated with MEK inhibitors (66-68), and indicative of in vivo efficacy (64). These findings suggest that trametinib sensitive HS cell lines are more dependent on activated MAPK signaling, whereas HHS DH82 cells are able to evade apoptosis under MEK inhibition, likely by an alternative mechanism of survival.

Rescue from apoptosis induced by trametinib has been previously linked to a hyperactivated PI3K-AKT pathway, counterbalancing the inhibition of the MAPK pathway (64, 69, 70). Indeed, our results suggest that the PI3K/AKT pathway was upregulated in DH82 cells, as indicated by the lack of PTEN expression and overexpression of p-AKT. We hypothesize that in DH82 cells, PI3K/AKT is a major driver. This mechanism of resistance was demonstrated when the MEK inhibitor-sensitive status of KRAS mutant cells was reversed through PTEN depletion (70). Although the role of the PI3K/AKT pathway in HS is unknown, formation of HS was observed in PTEN mutant mice, suggesting its role as contributor for tumorigenesis (71). Comparison of such pathways in HS may help delineate sensitivity to MEK inhibitor-based treatment, and indicate targets for alternative drugs, i.e. AKT inhibitors.

In order to evaluate our in vitro findings in vivo, we used a mouse model that initially presented an intrasplenic tumor that later spread to multiple intra-abdominal organs, and thus, can be considered an orthotopic model of a metastatic/disseminated form of HS, the most clinically challenging one due to the lack of specific systemic treatments. In this model, trametinib significantly suppressed tumor growth (p=0.02 - Student t-test) and increased survival time (p=0.002 - Mantel Cox test). Untreated mice reached humane endpoints and were euthanized between days 21 and 30 post-cells injection, while treated mice survived beyond 37 days and were alive by the end of the study. Similar findings
were observed in BRAF and KRAS mutant cancer xenograft models with inhibition of tumor growth at doses ranging from 0.1mg/kg to 1mg/kg once daily orally (48, 64, 72, 73). A marked drop in the bioluminescent signal in some mice at the final reading is likely associated with poor vascularization and central necrosis within the tumor, limiting the substrate and oxygen that generate the bioluminescent signal (74). Although trametinib is an oral drug, the IP route for treatment was chosen as a reasonably easy method to administer the drug, minimizing the stress to the animals. Moreover, we anticipated that when given IP, trametinib should be mostly absorbed through the surface of membranes into the portal vein (75). Due to the drug’s long half-life, steady state was certainly achieved at day 23 of treatment when average plasma concentration was 21.4nM (17-25nM), and tissue concentration was 30.2nM (5-53nM), which are higher than the IC50 of most of cell lines. Pharmacokinetic studies reported for drug approval showed that mice treated orally with 1mg/kg daily had significantly higher plasma concentrations (459nM) (76). Although the route chosen was different from the actual route of administration in patients, our results are within an achievable concentration seen in human patients treated with trametinib at the regular dose scheme (2mg/day), reported as steady state concentrations of 19.3-19.6nM (77, 78), and 34nM (54). Moreover, we demonstrated that treatment with trametinib resulted in a sustained target engagement, with significant downregulation of p-ERK in tumor tissues after 24hs of dosing. Similarly, other studies have reported that trametinib caused inhibition of p-ERK in xenograft tumors of colorectal cancer for more than 8hs after a single dose (64), and over 24hs when administered PO for 7 days in a model for melanoma (48). Higher plasma levels of trametinib (>300nM) were reported in mice at doses of 3mg/kg/day PO (48), and thus additional studies with even higher doses of trametinib can be undertaken. However, our findings do indicate that trametinib can be an effective treatment for HS.

As we used humane endpoints for decisions for euthanasia, control mice were euthanized at different time points, as the disease advanced. Due to the variable time points of euthanasia, the metastatic rate could not be compared across groups. When metastatic foci were further studied, we observed
smaller metastatic foci and a less compromised hepatic architecture in the liver of mice in the
treatment group as compared to the control group (Supplementary Figure S2), suggesting that
trametinib may have inhibited growth at metastatic sites. Untreated control mice had a diffuse
metastatic pattern and abnormal architecture of the liver with higher level of cell necrosis, which may
be associated with an impaired hepatic function, as reflected in the higher plasma liver enzyme
concentrations. However, the difference in liver function could also be related to the fact that
euthanasia was performed at the end stage of all untreated mice, whereas it was done to treated
mice at a predetermined time when the study ended. Therefore, an impaired liver function could also
be related to the moribund state of untreated mice rather than tumor burden. Treatment alone did not
seem to have an effect on liver function as no difference was observed between untreated naïve mice
and treated mice. Untreated naïve mice had significantly higher levels of albumin when compared to
either vehicle control or trametinib treatment groups, indicating that the lower levels of albumin in the
vehicle control and the treatment group are likely caused by the presence of the xenograft tumor,
decreasing liver tissue and affecting the liver function as well as a poor body condition and
malnutrition due to their moribund state.

Our results suggest that a dysfunctional MAPK pathway plays a role in canine HS, and that targeting
MEK is a promising therapeutic strategy. Two of the four canine cell lines carry previously described
mutations in genes associated with the activation of MAPK pathway, PTPN11 and KRAS. These
mutations can be potentially used as indicators of sensitivity to trametinib, however, a larger number
of cases are needed before this can be confirmed. We hypothesize that additional activating
mutations are present in the oncogenic MAPK cascade in canine HS and can be targeted by
trametinib. Thus tumor sequencing studies are needed to unravel potential driver mutations, in these
and other genes, which are currently underway in our laboratory. Understanding other activated
signaling pathways such as PI3K/AKT, may help predict sensitivity or resistance to MEK inhibition,
and identify other therapeutic targets.
In conclusion, trametinib represents a novel targeted therapy for HS in dogs. Clinical trials in human patients with HS are challenging as case accrual would take an extended time due to the low incidence of HS. Dogs, on the other hand, present with an appreciable frequency of HS, and therefore represent an important translational model and can contribute with valuable information regarding mechanisms of tumorigenesis, novel targets for therapy, and provide proof of concept studies that can translate to humans. Clinical trials to test the safety and efficacy of trametinib in canine patients with HS are warranted to further generate relevant information in this large model of spontaneously occurring cancer.
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Figure Legends

Figure 1: Trametinib causes cell cycle blockage and promotes apoptosis in sensitive HS cell lines

A) Cell viability was measured using the CellTiter Glo assay on three HS cell lines (BD, OD, PJ), a hemophagocytic HS cell line (DH82) and two normal dog fibroblast cell lines (FB BMD, FB) treated with trametinib for 72hs. B) Cells were treated with either vehicle, 1nM, 10nM, 100nM or 1μM trametinib for 24hs, and analyzed for DNA content based on propidium iodine uptake. Trametinib blocked cell cycle progression and triggered G1 phase arrest in all cancer cell lines BD, OD, PJ and DH82, but not in normal fibroblasts. Statistical analysis was done using Tukey’s multiple comparison two-way ANOVA test. C) Level of apoptosis was measured using Caspase-Glo 3/7 on cells treated with either vehicle, 1nM, 10nM, 100nM or 1μM trametinib, or staurosporine (positive control) for 24hs. Apoptosis was significantly increased in the cell lines that are sensitive to trametinib (BD, OD and PJ), while the effect was not observed in the resistant cell lines (DH82 and FB). Data were statistically analyzed using a multiple comparison two-way ANOVA test. For all assays (A, B and C), values correspond to averages and error bars to standard deviations generated from three experimental repeats.

Figure 2: MEK inhibitor trametinib downregulates downstream p-ERK in a dose dependent manner

Levels of proteins of cells treated with trametinib for 2hs were analyzed using Western-Blots. A) In all cell lines, the level of p-ERK decreased as the concentration of trametinib increases. Expression of pERK was significantly reduced at trametinib concentrations of 10^-8 M, and undetectable at 10^-7 M. B) Increased expression of p-Akt and decreased expression of PTEN were observed in the resistant hemophagocytic HS cell line DH82. There was no difference in expression of other components of the MAPK pathway.

Figure 3: Trametinib decreases the rate of tumor growth and increases survival time of xenograft mice injected intrasplenically with BD-luc cells.

Immunocompromised mice were injected into the spleen with 1.5x10^6 BD-luc cells. After 14 days, mice were randomized in two groups to receive daily treatment with either 1mg/kg trametinib or vehicle IP. Animals were euthanized as humane endpoints were reached, or after 20 days of treatment. The intensity of the bioluminescence signal of mice from the vehicle group increased more
rapidly than the intensity of the signal in the treatment group. Images were taken biweekly using IVIS as shown in few representative pictures of different stages of treatment (A), and graphically (B). The values of each group of mice were statistically different based on Student t-test analysis (thicker lines represent mean values of each group). The Kaplan-Meier survival curve shows that trametinib increased survival time of mice (C), and the difference was statistically significant based on Mantel-Cox test.

**Figure 4: Trametinib decreases activity of MAPK signaling in tissues, where the drug was present at concentrations consistent with therapeutic levels and mitigates liver injury associated with tumor burden**

(A) Expression of p-ERK and total ERK was measured in post-mortem tissues from mice, where each column represents one mouse. Trametinib significantly decreased p-ERK of splenic tissues of treated mice. Statistical analysis used was Mann-Whitney one-tail t test (p-ERK was normalized with values of tERK). (B) Samples of different organs and plasma were collected post-mortem 24hs after last dosing and analyzed for the concentration of trametinib. Data points represent individual mouse, identified by a specific number (1-5). Trametinib was consistently found in splenic tissues. It was also found in other tissues and plasma at variable concentrations. Bars represent mean values and standard deviations. (C) Levels of albumin and liver enzymes were determined from plasma samples of mice post-mortem. Mice bearing tumors presented significant lower levels of albumin than naïve non-tumor bearing mice. Mice from the vehicle control group had significant higher levels of ALT, when compared to non-tumor bearing mice and trametinib treated mice. AST levels were not different across the groups. (D) Histologically, splenic tumors consisted of diffuse infiltrates of highly pleomorphic, neoplastic round cells, with marked anisocytosis, anisokaryosis, and numerous multinucleated cells. Variably sized, nodular accumulations of neoplastic cells were also present within the liver. Neoplastic cells within the spleen and hepatic metastatic foci had strong positive cytoplasmic immunoreactivity for CD204. (E) Untreated mice had a significantly higher level of cell necrosis within their splenic and hepatic tissues, based on a semi quantitative score system (52).
Figure 1

A

![Graph showing cell viability against Trametinib concentration.](image)

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<tr>
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B

![Bar charts comparing cell cycle distribution under different conditions.](image)

C

![Bar charts showing caspase 3/7 activity fold increase.](image)
Figure 2

A

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Figure 3

A

Days of treatment

Treatment Group

Vehicle Group

Day -6

Day -1

Day 6

Day 9

Day 13

Day 20

B

Spaghetti plot

Days of treatment

0 2 4 6 8 10 12 14 16 18 20

Boliminescence Signal

5x10^10

4x10^10

3x10^10

2x10^10

1x10^10

0

Trametinib

Vehicle Control

p = 0.02

Days post injection

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38

C

Kaplan-Meier Survival Curve

Percent

100

80

60

40

20

0

Trametinib

Vehicle Control

p = 0.002

Days post injection

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Figure 4

A

![Image of Western Blot with Vehicle Control and Trametinib for pERK and ERK in Spleen]

B

![Bar chart showing Trametinib Concentration (nM) for different tissues: Muscle, Heart, Brain, Kidney, Spleen, Liver, Plasma]

C

![Box plots for Albumin (g/dL), ALT (U/L), AST (U/L)]

- Untreated not bearing tumors
- Vehicle Control
- Trametinib

- * p ≤ 0.05
- ** p ≤ 0.01
- *** p ≤ 0.001

D

- H&E
- CD204

Spleen

E

Necrosis Score (average score from 5 mice/group)

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Semi quantitative score system

1 = 1-5% necrosis
2 = 6-40% necrosis
3 = 41-80% necrosis
4 = >80% necrosis
Molecular Cancer Therapeutics

Targeting MEK in a translational model of histiocytic sarcoma

Marilia Takada, Jeremy M.L. Hix, Sarah Corner, et al.

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