EVALUATION OF THERAPEUTIC RESPONSE OF HISTIOCYTIC SARCOMA CELL LINES TO NOVEL SMALL MOLECULE INHIBITORS OF RECEPTOR TYROSINE KINASES

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ABSTRACT

EVALUATION OF THERAPEUTIC RESPONSE OF HISTIOCYTIC SARCOMA CELL LINES TO NOVEL SMALL MOLECULE INHIBITORS OF RECEPTOR TYROSINE KINASES

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The current standard of care treatment for canine histiocytic sarcoma (HS) is based on the administration of conventional chemotherapeutic drugs, which results in low percentage of partial and short-term favorable responses. In order to identify novel drug candidates for the treatment of dogs with HS, we investigated the cytostatic activity of a panel of sixteen compounds over two canine HS cell lines. Our results demonstrated that dasatinib, a receptor tyrosine kinase pan-inhibitor, and other novel molecularly-targeted drugs JQ1, a BET bromodomain inhibitor, and bortezomib, a proteasome inhibitor, effectively inhibited the growth of HS cells *in vitro*. The antiproliferative response of dasatinib was augmented when combined to doxorubicin, a classical chemotherapeutic agent. For all these drugs, the effective inhibitory concentration *in vitro* was within a clinically achievable and tolerable plasma concentration *in vivo*, as described in the veterinary and human medicine literature. In this study we identified three molecularly targeted drugs that may represent a promising anticancer strategy for canine HS. Further *in vivo* studies and clinical trials are needed to fully evaluate therapeutic potential of these drugs in HS in dogs and in similar disorders in humans.

DEDICATION

Dedicated to my father Kinichi Takada.

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Thank you my family. Nala who brought so much joy. Leo and Emy, your my inspiration and my best friends. Mama, your sweetness and love give me strength to keep following my dreams. Papa, thank you for teaching me to be ambitious and to enjoy life as much as I can.

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KEY TO SYMBOLS or ABBREVIATIONS

ALK = anaplastic lymphoma kinase

ALL = acute lymphocytic leukemia

AML = acute myeloid leukemia

ATP = adenosine triphosphate

BMD = bernese mountain dog

CCNU = *N*-(2-chloroethyl)-*N*'-cyclohexyl-N-nitrosourea

CML = chronic myelogenous leukemia

CMML = chronic myelomonocytic leukemia

CNA = copy number aberration

DC = dendritic cell

DMSO = dimethyl sulfoxide

DNA = deoxyribonucleic acid

EGFR = epithelial growth factor receptor

EPHR = ephrin receptor

FDA = food and drug administration

FGFR = fibroblast growth factor receptor

GIST = gastrointestinal stromal tumor

GM-CSF = granulocyte-macrophage colony-stimulating factor

GTPase = guanosine triphosphate

HS = histiocytic sarcoma

IL-1α = interleukin 1 alfa

IL-1β = interleukin 1 beta

IMHA = immune mediated hemolytic anemia

M-CSF = macrophage colony-stimulating factor

MCT = mast cell tumor

NGFR = nerve growth factor receptor

NOAEL = no-observed-adverse-effect level

PAHS = peri-articular histiocytic sarcoma

PBMC = peripheral blood mononuclear cells

PDGF = platelet growth factor

PDGFR = platelet growth factor receptor

Ph+ CML = Philadelphia chromosome positive chronic myelogenous leukemia

RCC = renal cell carcinoma

RTK = receptor tyrosine kinase

SCF = stem cell factor

TCC = transitional cell carcinoma

TD = therapeutic dose

TGF-β = tumor growth factor beta

TNF-α = tumor necrosis factor alfa

VEGFR = vascular endothelial growth factor receptor

CHAPTER 1

Introduction

Canine histiocytic sarcoma is a malignant tumor of histiocytes that is commonly seen in specific canine breeds including Bernese Mountain Dogs (BMD) and Flat-coated Retrievers.[1, 2] In a recent study, a region of canine chromosome 11 was found to be associated with increased susceptibility to HS in a population of BMDs, and some data was presented that suggest dysregulation of the tumor suppressor genes *MTAP/CDKN2A*/*B* located in this region.[3, 4] However, other association studies carried out in a US BMD population has not confirmed this association (Dr. Yuzbasiyan-Gurkan, personal communications). Because of its aggressive and metastatic nature, dogs with HS are invariably treated systemically with chemotherapeutic agents. However, treated dogs respond poorly and temporary to the current standard of care, the cytotoxic drug *N*-(2-chloroethyl)-*N*'-cyclohexyl-N-nitrosourea (CCNU).[5, 6] A few clinical studies on dogs with HS reported alternative medical strategies; still no effective one has been identified.[7-10]

Molecularly-targeted drugs, such as inhibitors of tyrosine kinase receptors, are part of a new generation of agents that disrupt specific targets on signaling pathways that malignant cells rely on to grow and survive.[11] Receptor tyrosine kinases (RTKs) are important molecular targets in this scenario, because of their fundamental role on sustained proliferative signaling in cancer cells.[12] The deregulation of RTKs caused by receptor overexpression, mutation or structural re-arrangements has been reported in dogs with cancer, and it has been correlated with tumor behavior, prognosis and

response to treatment.[13, 14] One example is the mutation in the *c-kit* gene on a significant proportion of canine mast cell tumors (MCTs), that seems to be associated to tumors with higher grade, increased risk of local recurrence and poor outcome.[15, 16] The small molecule veterinary-approved, KIT inhibitor toceranib (Palladia®, Pfizer) benefited dogs with recurrent MCT that failed previous treatments. Dogs carrying the *c-kit* mutation were twice as likely to respond to toceranib than those with wild-type *c-kit* (67% vs. 37%).[17, 18] Interestingly, there is evidence to support the use of toceranib in a variety of solid tumors in dogs (i.e.; anal sac gland adenocarcinoma, thyroid carcinoma, head and neck carcinoma, nasal carcinoma, mammary carcinoma, and osteosarcoma).[14, 17, 19] This versatility is most likely due to the multi-target characteristic of most tyrosine kinase inhibitors (TKIs), allowing to extend their use to multiple malignancies.[20, 21] Previous studies combined TKI and traditional chemotherapy agents for treatment of dogs with MCT. This strategy has shown that this therapeutic approach seems to be effective and well-tolerated for the patient.[22, 23]

Dogs with HS carry a poor outcome because of the absence of an effective therapeutic option, and may also be benefit from molecularly-targeted anticancer strategy. The therapeutic potential of TKIs for dogs with HS was evidenced in a study *in* vitro, where dasatinib, a tyrosine kinase pan-inhibitor, was effective against multiple canine HS cell lines.[24] Our goal, in the present study, was to further explore the potential of TKIs for the treatment of dogs with HS.

For those malignancies such as canine HS, where the disease driver molecular targets are unknown, the search for small molecular inhibitors with therapeutic potential can be carried out through a drug-screening assay. [24, 25] In the present study, we

tested a collection of 16 compounds, including TKIs, cytotoxic chemotherapeutic drugs, and novel molecularly-targeted drugs, over two canine HS cell lines, with the objective to identify drug candidates that target molecular pathways underlying canine HS oncogenesis.

CHAPTER 2

Literature Review

2.1. HISTIOCYTIC SARCOMA (HS) IN DOGS

2.1.a. Epidemiology of canine HS

Histiocytic sarcoma is an uncommon neoplasm, representing <1% of all cancers in dogs.[26] However, it is commonly seen in specific breeds, especially in the BMD that carries an incidence of at least 25%.[1] Flat-coated Retriever is also a predisposed breed for HS, which accounts for 25% of their tumors. Other overrepresented breeds are the Golden Retrievers and Rottweilers. Males and females are equally affected, and dogs are often middle aged or older (8-10 years).[2, 27-30] There is no report of neither environmental nor dietary risk factors involved in the development of HS in dogs.[18]

The high prevalence of HS in specific breeds may suggest the existence of heritable risk factors that have influence in the development of the disease. An epidemiologic investigation on a large population of BMD showed that most of the affected dogs (70%) had relatives with a confirmed diagnosis of HS. Analysis of the disease distribution between these families could not be explained by a fully recessive model, so the authors suggested an oligogenic model to best describe the genetic pattern behind this disorder.[1]

A study looking at the molecular cytogenetic characterization of HS from BMD and Flat-coated Retrievers assessing DNA copy number aberrations (CNAs) demonstrated that most of the CNAs were shared between the breeds, suggesting that they are associated more with the cancer phenotype than with the breed.[4] Although some

studies suggest the connection between the susceptibility to HS to the deregulation of particular genes (*CDKN2A/B*, *RB1*, and *PTEN*), the genetic profile promoting oncogenesis in HS is not yet clear.[3, 4] Studies involving gene expression and mutation analysis have also been conducted for KIT, FLT3 and MET, as well as tumor necrosis factor-α (TNF-α), IL-1α, and IL-1β, however no significant results were reported.[31, 32]

2.1.b. Pathology of canine HS

Histiocytic sarcoma is a malignant tumor of proliferative histiocytes, a class of cells arising from macrophage and dendritic cell [33] lineage.[33] Both myeloid dendritic antigen presenting cells and macrophages, evolve from a common hematopoietic $CD34^+$ stem cell precursor in the bone marrow. Under the influence of a variety of growth factors and cytokines, the progenitor cells develop into either DC or macrophages. The development of DC can be influenced by FLT3 ligand, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-4, and tumor growth factor-β (TGF-β). While the differentiation to macrophages are stimulated by GM-CSF and macrophage colony-stimulating factor (M-CSF). [34, 35]

Histiocytic sarcoma in dogs can present as either a localized or a disseminated disease, depending if the tumor is confined into a single site or if multiple organs are involved.

Localized histiocytic sarcoma is locally invasive, and commonly metastasizes to satellite lymph nodes and other organs, with a reported high metastatic rate of 70-91%.[6, 28, 36, 37] This form most frequently involves the skin and subcutis of the dog

extremities, and peri-articular tissues of appendicular joints, but it can also be found in the spleen, lymph nodes, lung, and bone marrow.[36, 38]

Peri-articular histiocytic sarcoma (PAHS) is the most common synovial tumor in dogs, and despite its potential of spread to other organs (63%), dogs with PAHS carry a better outcome in comparison to other locations, especially if treated in the early stage of the disease. Usually one joint is affected but the involvement of multiple joints has been reported. PAHS seems to be more frequently located either at the stifle or elbow joints in the dog.[28, 36, 39] It has been suggested an association between previous inflammatory joint disease and the development of PAHS based on the rationale of tumor-promoting inflammation.[28, 39]

The disseminated form of HS, also referred to as malignant histiocytosis, is a multisystem disease, and although it can affect essentially any organ in the body, is primarily localized in the spleen, lungs, bone marrow, liver and lymph nodes. This form of the disease could indicate metastasis and progression of the localized form. [36, 38] BMD seem to be more susceptible to the disseminated form of HS than flat-coated retrievers, which have a higher prevalence of localized form, especially in the skin and subcutis areas of the limbs.[3, 37]

Hemophagocytic histiocytic sarcoma is referred to a subtype of histiocytic sarcoma that it is thought to originate from the splenic red pulp and bone marrow macrophages, instead of interstitial DC, as are most of the other types of HS in the dog. It carries a highly aggressive clinical course, and it distinguishes from the other forms because of the marked erythrophagocytosis present in the affected organs, resulting in a significant non regenerative anemia.[40]

2.1.c. Clinical presentation of canine HS

The initial clinical presentation of dogs with HS may differ according to the location of the primary tumor and also the extent of the disease, but in most cases dogs present with nonspecific clinical signs such as lethargy, inappetence, weakness, weight loss, visible mass, lameness, cough, vomiting, and lymphadenomegaly.[6, 38, 41]

The commonly reported clinicopathologic abnormalities at presentation include anemia, thrombocytopenia, neutrophilia with or without left shift, hypoalbuminemia, hypercalcemia, and increases in liver enzyme activity.[1, 5, 6]

The diagnosis of this subtype of histiocytic sarcoma in some cases is misinterpreted as immune-mediated hemolytic anemia (IHMA) because of the similarities in the clinical presentation between these two entities.[40]

2.1.d. Diagnosis and staging of canine HS

The definitive diagnosis of histiocytic sarcoma in dogs is challenging because of the pleomorphic nature of the neoplastic cells, which may confer similar morphologic characteristics to carcinomas and round cell tumors.[18]

The different forms of HS are not distinguishable in the histopathologic examination of the tumor tissues. The description can include features such as poorly demarcated, locally invasive tumor masses, heterogeneous population of cells including histiocytic-spindle-pleomorphic cells, multinucleated histiocytic giant cells, erythrophagocytes, and lymphocytes. HS cells may display marked anysokaryosis and anysocytosis, and mitotic figures are commonly present. Their nuclei are round, oval, or reniform with prominent nucleoli, and their cytoplasm is moderate to abundant, lightly basophilic, and vacuolated.[1, 27, 38]

Immunohistochemistry analysis is crucial to support a definitive diagnosis of HS in dogs. A panel of expected expression patterns to leukocyte-specific antibodies has been proposed using snap-frozen and formalin-fixed tissues **(Table 2.1 and 2.2)**.[38, 42] However, at our institution a pattern of CD3−, CD79a− and CD18+, is considered enough to establish the diagnosis of HS, which is in agreement with several studies.[1, 36, 38, 39]

Table 2.1. Immunohistochemical expression patterns for HS in dogs

Tissue type	Expression pattern
Snap-frozen	CD1+ CD4− CD11c+ CD11d− MCH II+ ICAM-1+ Thy-1+/−
Formalin-fixed	CD3− CD11d− CD18+ CD45RA− CD79a− E-cadherin−

Table 2.2. Immunohistochemical markers for specific cellular antigens

Marker	Cell type
CD1	Langerhan's cells, DC
CD3	T lymphocytes
CD4	Helper T lymphocytes, activated DC
CD11c	DC, natural killer cells
CD11d	Macrophages
CD18	Broad staining of leukocytes, prominent in histiocytic tumors
CD45RA	B lymphocytes and naïve T lymphocytes
CD79a	B lymphocytes
ICAM-1	DC
MHCII	Antigen presenting cells
Thy-1	CD34+ hematopoietic cells, specifically interstitial DC
E-cadherin	Specific to epidermal Langerhans cells, epithelial cells

As mentioned previously, the cells of origin of hemophagocytic HS are the macrophages rather than DC, consequently, this subtype of HS can be distinguished by positive diffuse (perimembrane) expression of CD11d, a specific marker for macrophages. Then the pattern of expression consists of CD18+, CD11d+, CD11c−, MHCII+, and minimal expression of CD1.[40, 43]

2.1.e. Treatment and prognosis of canine HS

The two forms of HS might have distinct clinical courses, with the localized form of HS progression slower than the disseminated form, however because of the extremely high metastatic rate of 70-91%, all dogs invariably carry a poor prognosis, reflected in the reported median survival time of 2-4 months, despite any form of treatment.[5, 28, 37, 38]

Although PAHS has a similar metastatic potential, it seems to carry a more favorable outcome when patients are submitted to multimodality treatment. Dogs with PAHS that received treatment, had a significant longer median survival time of 391 days in comparison with 128 days from treated dogs with non-PAHS.[36] The median survival time decreased to 61 days when the vast majority of dogs with PAHS didn’t receive any form of treatment.[39]

Treatment options for histiocytic sarcoma in dogs may vary from complete surgical excision of the tumor, local radiation therapy, to different chemotherapy drugs. Although there is no evidence that a single modality of treatment is more beneficial than others, patients with HS are all recommended to receive systemic therapy because of the high metastatic potential. [6]

The most effective chemotherapeutic agent against HS in dogs is CCNU, a cytotoxic drug that provides a response rate ranging from 29 to 46% for a median of 85-96 days.[5, 6] A preliminary study of dogs with HS using a distinct conventional chemotherapeutic drug doxorubicin, showed a similar response rate of 46% for a median of 93 days.[8] A few additional clinical studies tested different medical interventions for this disease, though no effective option has been yet identified. Clinical reports in the literature demonstrated potential therapeutic value of liposomal doxorubicin, paclitaxel, liposomal clodronate, and also immunotherapy using human cytotoxic T-cell line (TALL-104).[7, 9, 10, 44] Studies *in vitro* have shown promising results using novel therapeutic approaches including TKIs, and the combination of biphosphonates and conventional chemotherapeutic agents.[24, 44] However, further studies are warranted to confirm the clinical therapeutic potential of these drugs.

Hemophagocytic HS in dogs appears to behave more aggressively. Regardless of any therapy, affected dogs live for only 1 to 2 months.[40, 41]

In the majority of cases, the diagnosis of HS is established when the disease is advanced and, when the tumor is disseminated to multiple organs. Although it is not clear whether early detection of HS could improve the patient's outcome, the identification of biomarkers associated with canine HS potentially benefit especially dogs from predisposed breeds.[45, 46]

2.2. PROTEIN TYROSINE KINASE RECEPTORS

2.2.a. Structure of receptor tyrosine kinases(RTKs)

Protein tyrosine kinase receptors are transmembrane-spanning receptors located in the cell membrane, that have an intrinsic protein tyrosine kinase activity that is normally

dependent on binding of the cognate ligand.[13] There are 58 known RTKs distributed within 20 different families. Each member has a specific biological function, however the different families of RTKs are morphologically related and share a common structure consisting of a large ligand-binding extracellular domain, a lipophilic transmembrane-spanning region, and a cytoplasmic domain. **(Figure 2.1)**. [13, 47, 48]

The morphology of the extracellular domain varies between the different type of receptors, and determines the proper recognition and binding to a wide variety of specific ligands. The plasticity of this region is a result of combinations of cysteine-rich motifs, immunoglobulin-like repeats (Ig-like), fibronectin type III repeats (FNIII), and EGF motifs. [49]

The transmembrane domain anchors and stabilizes the receptor in the plasma membrane. It functions as a communication bridge between the extracellular environment and the internal compartments of the cell.

The structure of the cytoplasmic portion is composed of a catalytic tyrosine kinase domain, a juxtamembrane region and a C-terminal tail.

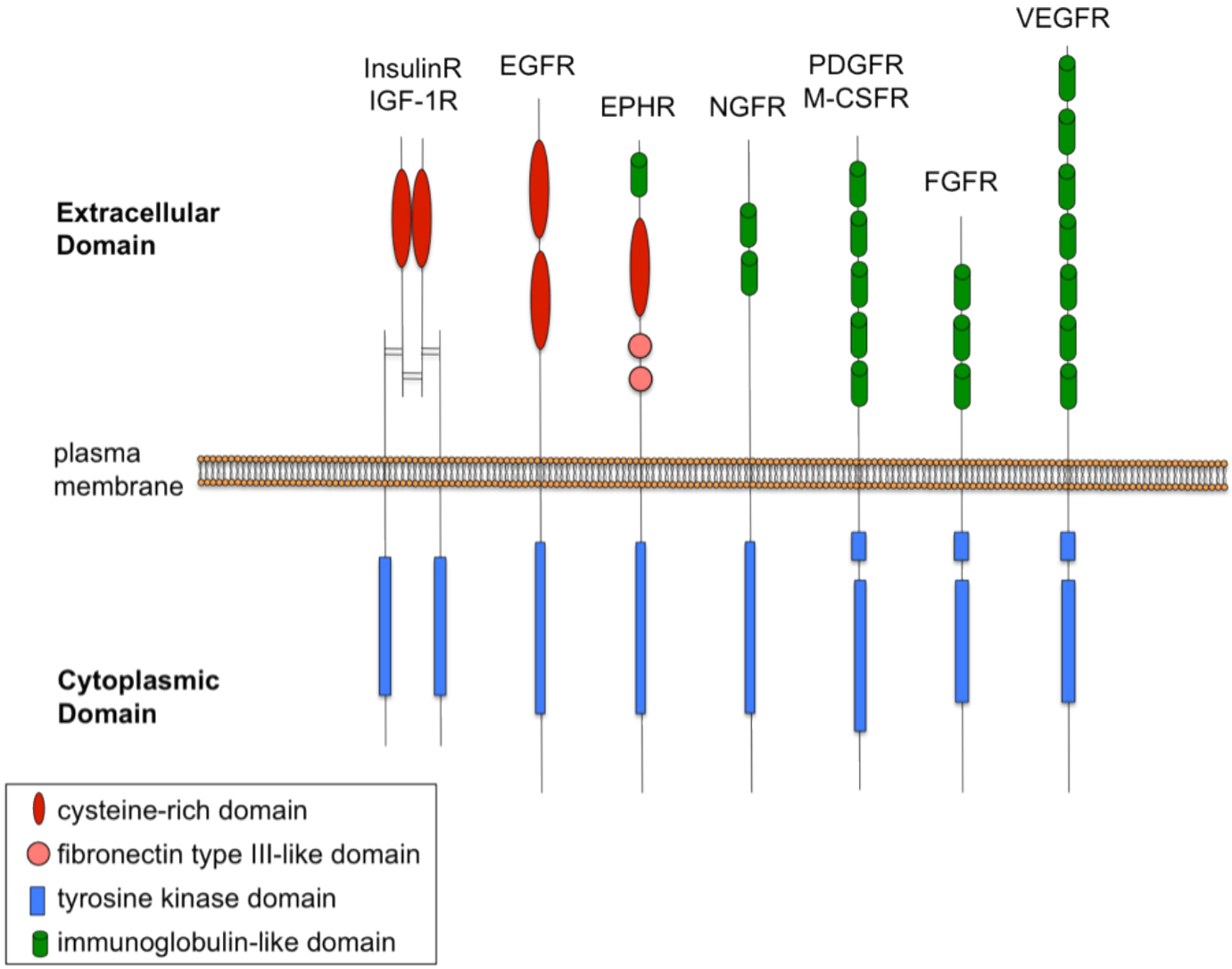
Tyrosine kinase domain has the highest level of conservation among tyrosine kinase receptors, and its integrity is essential for adequate receptor signaling. This domain contains an ATP-binding site that catalyzes autophosphorylation of tyrosine residues of the receptor. [50]

The juxtamembrane sequence that separates the transmembrane domain and the cytoplasmic domain, is well conserved between members of the same receptor family, although it diverges between different families. This domain acts by modulating the

receptor activity under stimuli originating outside the receptor itself, a heterologous stimuli (transmodulation) and in some instances, acts as a negative regulator.

The most variable region between tyrosine kinase receptors is the C-terminal tail, which contains numerous tyrosine residues that are phosphorylated by the activated kinase. [49]

Figure 2.1. Families of receptor protein-tyrosine kinases (Modified from Weinberg RA, 2014)



* For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

2.2.b. Functions and mechanisms of activation of RTKs

RTKs have an important role in mediating the transduction of extracellular signal into the intracellular environment. These signals are involved in a wide range of important cellular processes such as cell growth, cell proliferation, differentiation, and apoptosis. In normal cells, this regulatory activity must be tightly controlled and balanced, sustaining an adequate behavior and function of the cell.

In the absence of a ligand, RTKs are inactive, and presents as a monomeric and unphosphorylated structure. In the state of inactivity, the tyrosine kinase domain has a particular conformation that assures a low catalytic activity of the receptor. Moreover, an interaction between the juxtamembrane and the tyrosine kinase domains has an inhibitory effect to the enzyme, and therefore, helps to sustain a low catalytic condition of the receptor.[51]

The activation of a RTK starts when a growth factor molecule binds to its ligand-binding extracellular domain, which leads to the oligomerization of the receptor with a second monomer receptor. In the dimerized stated, the autoinhibitory activity of the juxtamembrane domain from the receptor is disrupted. Next, a process of transphosphorylation occurs between the two receptors, where each tyrosine kinase domains catalyzes the transfer of γ-phosphate group from adenosine triphosphate (ATP) to tyrosine residues in the C-terminal cytoplasmic tail of the other receptor. In this way, binding sites are generated for signaling proteins that are recruited to the cell membrane, activating a cascade of downstream intracellular signaling pathways. Intracellular mediators transduce those signals through the cytosol and into the nucleus, where the expression of genes is affected. Those genes are involved on a variety of

biological processes, including cell growth, migration, differentiation, and cell death.[13, 51, 52]

The receptor oligomerization can occur between two identical receptors (homodimerization), different members of the same receptor family, or in some cases, between a receptor and an accessory protein (heterodimerization). Heterodimerization optimizes the receptor activity by increasing the repertoire of possible ligands recognized by the receptor. [49]

The activity of a tyrosine kinase receptor is terminated by phosphatases that hydrolize tyrosil phosphates, and by inhibitory feedback mechanisms induced from downstream pathways. [51]

2.2.c. Deregulation of RTKs in cancer

Normal cells are constantly under growth-stimulatory signals from their surroundings, which are processed and transmitted internally, and ultimately determine whether cells should growth and divide or not. On the other hand, cancer cells can acquire the ability of sustain proliferative signaling through several mechanisms such as the deregulation of protein tyrosine kinases activity, since they play an important role as regulators of proliferative and survival signal transduction pathways **(Table 2.3)**. This is evidenced in the fact that more than 50% of tyrosine kinase receptors have been found to be either overexpressed or mutated in human malignancies.[12, 13]

Table 2.3. Tyrosine kinase receptors associated with cancer [18]	
Tyrosine Kinase	Cancer Association
EGFR	Breast, ovary, lung, stomach, colon, glioblastoma
Insulin receptor	Sarcomas, cervix, kidney
PDGFR	Glioblastoma, ovary, CMML, GIST
KIT	AML, GIST, seminoma, MCT, melanoma
Flt3	AML
VEGFR	Kaposi's sarcoma, hemangiosarcoma, melanoma
FGFR	AML, lymphoma, breast, prostate, multiple myeloma, TCC
NGFR	Thyroid cancer, neuroblastoma, fibrosarcoma, AML
Met/Ron	Thyroid cancer, osteosarcoma, rhabdomyosarcoma, liver, kidney, colon
EPHR	Melanoma, stomach, colon, breast, esophagus
AXL	AML
Tie	Stomach, hemangioblastoma
RET	Thyroid cancer, multiple endocrine neoplasia
ALK	Non-Hodgkin's lymphoma, lung

The constitutive proliferative signaling from RTKs involved in cancer occurs as a result of several transforming mechanisms, including:

i. Overexpression of receptor by abnormal number of RTKs

In comparison to normal cells, cancer cells may greatly exceed the amount of RTKs on their cell membrane. As RTKs can move freely on the cell surface, when present in a higher number, they frequently collide to themselves, causing accidental events of transphosphorylation and consequently, receptor activation. In addition, cancer cells might become more sensitive to the signal from growth factor molecules, and so they can be activated even in the face of a low level of growth factor molecules.[13]

ii. Mutations in the RTK gene

Cancer cells can carry a variety of mutations in RTK-encoding genes, including those that cause amino acids substitutions on the transmembrane domain, or truncations in the ectodomain (gene fusion) and cytoplasmic catalytic domain (particularly ATP-binding motif). RTKs with these forms of mutations are considered oncogenics because they are constitutively dimerized and activated, even in the absence of ligand binding.[13] The dependency of the cell to the continuous signal from transformed kinases is called oncogene addiction, and makes the cancer cell particularly susceptible to the specific kinase inhbitors.[53]

iii. Autocrine signaling

Malignant cells may possess the capability of producing growth factors themselves, to which their receptors can bind and become activated, forming an auto-stimulatory signaling loop.[13]

iv. Deficiency on autoinhibitory mechanisms

Oncogenic mechanisms are often looked as activating factors however, they can also cause the suppression of normal autoinhibitory and regulatory processes in cancer cells. These negative-feedback mechanisms originating from downstream pathways function to balance intracellular proliferative signaling, and their disruption results in sustained proliferative activity of the cell.[12, 13]

2.2.d. RTKs as targets for cancer therapy

The crescent evidence that tyrosine kinase receptors play an important role on oncogenesis in a great variety of malignancies caused the emergent development of inhibitors of tyrosine kinase receptor for cancer treatment.

The first U.S. FDA approved small molecule tyrosine kinase inhibitor, imatinib mesylate (Gleevec®), emerged in the early 1990s as an inhibitor of the Bcr-Abl oncogene, which is carried by 95% of patients with chronic myeloid leukemia (CML), and 15 to 30% of those with acute lymphoblastic leukemia (ALL) as a result of t(9;22) translocation of Philadelphia chromosome (Ph). [54, 55] Newly diagnosed chronic phase CML patients had a significant improvement of outcome from treatment with imatinib, which caused a complete response of 76% at 18 months, in comparison to 15% ($P<0.001$) for patients treated with the conventional protocol of chemotherapy.[56]

Later on, imatinib showed to also inhibit receptor tyrosine kinases encoded by the c-*KIT* and platelet-derived growth factor receptor (PDGFR) oncogenes. Since activation mutations of these oncogenes were present in 70-80% and around 5% of the gastrointestinal stromal tumors, respectively, kit positive GIST patients were excellent candidates for imatinib therapy. [20] Indeed, the response to imatinib was 70% higher

than the response to conventional chemotherapy, increasing the range of patients that could have benefit from this drug. [21, 57]

The success with Gleevec encouraged attempts of creating other tyrosine kinase receptor antagonists known to play a role in the development of cancer. Currently there are 15 U.S. FDA approved small molecule tyrosine kinase inhibitors for the treatment of a total of at least 14 types of cancer **(Table 2.4)**

Table 2.4. U.S. FDA approved tyrosine kinase inhibitors for treatment of cancer in humans [58]		
Inhibitor	Target	Type of malignancy
Imatinib (Gleevec®)	c-KIT, PDGFR, Bcr-Abl	Ph+ CML, GIST, ALL
Dasatinib (Sprycel®)	Bcr-Abl, SRC, PDGFRβ, c-KIT, EPHR, EGFR	CML, ALL
Nilotinib (Tasigna®)	Bcr-Abl, c-KIT, PDGFR	CML
Bosutinib (Bosulif®)	c-Abl, Src-Abl, Bcr-Abl	CML
Lapatinib (Tykerb®)	HER1, HER2	Breast
Gefitinib (Iressa®)	HER1/EGFR	Non-small cell lung carcinoma
Erlotinib (Tarceva®)	HER1/EGFR	Non-small cell lung carcinoma
Vandetanib (Caprelsa®)	VEGFR, EGFR	Medullary thyroid
Vemurafenib (Zelboraf®)	B-RAFV600E	Melanoma
Crizotinib (Xalkori®)	Met, EML4-ALK	Non-small cell lung carcinoma
Sorafenib (Nexavar®)	C-RAF, B-RAF, c-KIT, FLT3, VEGFR, PDGFR	RCC, hepatocellular, prostate
Sunitinib (Sutent®)	FLT-3, VEGFR, PDGFR, c-KIT, RET, CSF-1R	GIST, RCC
Pazopanib (Votrient®)	VEGFR-1/2/3, PDGFR, FGFR, c-KIT, c-fms	RCC, soft tissue sarcoma
Regorafenib (Stivarga®)	c-KIT, Raf, VEGFR, Tie-2, PDGFR, RET	Colorectal
Cabozantinib (Cometriq®)	VEGFR, c-MET, FLT, Tie-2, c-KIT, RET	Medullary thyroid

2.2.e. Tyrosine kinase inhibitors (TKIs) in cancer therapy

Most of the tyrosine kinase inhibitor drugs have two different antineoplastic strategies. First, TKIs can directly suppress the catalytic activity of the tyrosine kinase by competing for the ATP binding site. The conformation of the kinase activation loop can be either active (type 1 - majority) or inactive (type 2). These inhibitors present one to three hydrogen bonds to the amino acids located in the hinge of the target kinase, therefore mimicking the hydrogen bonds that are normally formed by the adenine ring of ATP.[59, 60]

The second possible mechanism of action of TKIs is to block the oligomerization process by inhibiting the activation of fusion tyrosine kinases. [51, 61] Tyrosine kinase inhibitors drugs have a high specificity and a known complete distribution in tissues.[62]

2.2.f. Tyrosine kinase inhibitors in veterinary oncology

In 2009, toceranib (Palladia®) was the first U.S. FDA approved tyrosine kinase inhibitor for the treatment of cancer in dogs, specifically malignant canine mast cell tumor **(Table 2.5)**. As toceranib inhibits c-KIT RTK, dogs with mast cell tumor harboring the mutation in KIT particularly benefited from the drug, being twice as likely to respond than those without the mutation (67% versus 37%).[17] Later, toceranib was also applied for several other types of tumors in dogs such as anal sac gland adenocarcinoma, thyroid carcinoma, head and neck carcinoma, nasal carcinoma, and osteosarcoma.[19]

After toceranib, masitinib (Kinavet®) was the second tyrosine kinase inhibitor approved for the use in companion animals. This small molecule inhibits KIT, PDGFRα/β, and Lyn. In dogs with malignant mast cell tumor, masitinib significantly

improved the time to progression, and increased the number of patients with long-term disease compared to those treated with placebo (40% versus 15% alive at 2 years), particularly those patients carrying KIT mutation in their tumors. [63, 64] Cats with malignant mast cell tumor also showed response to imatinib treatment. [65, 66]

Table 2.5. U.S. FDA approved tyrosine kinase inhibitors for treatment of cancer in dogs [18]

Inhibitor	Target	Malignancy
Masitinib (Masivet®)	c-KIT, PDGFR, FGFR3, FAK pathway	MCT
Toceranib (Palladia®)	VEGFR, PDGFR, c-KIT, FLT3, CSF1R	MCT

Studies in dogs using tyrosine kinase inhibitors approved for human use have been pursued with some promising results. Imatinib showed antitumor activity against canine malignant mast cells in a mouse xenograft model. [67] A study from a Japanese group reported beneficial response in 10 of 21 dogs (48%) treated with daily dose of 10mg/kg of imatinib for 1-9 weeks. In contrast to toceranib and masitinib, no significant difference was observed on the response of dogs carrying c-kit mutation in exon 11 with imatinib. [68]

CHAPTER 3

Therapeutic Response of HS Cell Lines to RTK Inhibitors

3.1. INTRODUCTION

To date, there is no known effective targeted therapy against HS in dogs. The goal of this study was to evaluate the effect on cell growth of several TKIs that may target a critical pathway involved in the tumorigenesis of canine HS. In addition, conventional chemotherapeutic drugs, some which are routinely used for HS patients clinically, and novel targeted therapy drugs were also part of this experiment.

3.2. MATERIAL AND METHODS

3.2.a. Canine HS-derived cell lines

Two established HS cell lines were used: DH82 (CRL-10389™ - ATCC®, Manassas, VA) and BD (Michigan State University, East Lansing, MI). DH82 is a commercially available cell line, while BD is a cell line established in our laboratory from a histologically and immunohistologically confirmed histiocytic sarcoma in a BMD.

3.2.b. Cell culture conditions

DH82 cell line was grown in a cell culture flask containing EMEM ATCC® 30-2003 (Eagle's Minimum Essential Medium - ATCC®, Manassas, VA) while BD cell line was grown in RPMI 1640 (Roswell Park Memorial Institute - Life Technologies™, Carlsbad, CA). Both cell lines were supplemented with 15% heat-inactivated FBS (Fetal Bovine Serum - Life Technologies™, Carlsbad, CA), and 1% antibiotic-antimycotic 100X (Life Technologies™, Carlsbad, CA). The cell cultures were incubated at 37°C in a

humidified atmosphere of 5% CO_2, and for their maintenance, the medium was changed every 2-3 days, and confluent cells were subcultured every 3-5 days by detaching the cells with 0.25% Trypsin-EDTA 1X (Life Technologies™, Carlsbad, CA).

3.2.c. Chemotherapeutic drugs

For the drug-screening assay in HS cell lines, 16 different inhibitory compounds were tested **(Table 3.1)**. Stock solutions were prepared using the appropriate solvent for each drug, stored at -20°C, and protected from light. Serial dilutions of each test drug were prepared from these stock solutions in culture medium immediately before adding to the cells in such a way that the solvent concentration was always <1%. Each compound was tested at five to eight concentrations predicted to bracket the IC_{50} for that drug.

Table 3.1. List of the drugs used for the proliferation assays on HS cell lines

Name	Trade name	Solvent	Targets
Dasatinib	Sprycel	DMSO	Bcr-Abl, SRC-family, PDGFRβ, c-KIT, EPHR, EGFR (HER-1), HER-2
Erlotinib	Tarceva	DMSO	EGFR
Gefitinib	Iressa	DMSO	EGFR
Imatinib	Gleevec	DMSO	Bcr-Abl, PDGFRα, c-KIT
Masitinib	Masivet	DMSO	c-KIT, PDGFRα, -β, Lyn, FGFR3, FAK pathway
Nilotinib	Tasigna	DMSO	Bcr-Abl, c-KIT, PDGFRα, -β
Toceranib	Palladia	DMSO	VEGFR2, PDGFRβ, c-KIT
Sorafenib	Nexavar	DMSO	C-RAF, B-RAF, c-KIT, FLT3, VEGFR2, -3, PDGFRβ
Sunitinib	Sutent	DMSO	PDGFRα, -β, VEGFR1, -2, -3, c-KIT, RET, CSF-1R, FLT3
Tozasertib	none	DMSO	SRC, GSK3, FLT3, JAK2, Bcr-Abl
CCNU	Lomustine	Ethanol	Alkylation and cross-linking of DNA
Cladribine	Leustatin	DMSO	Purine analogue
Doxorubicin	Adriamycin	Saline	Inhibits DNA topoisomerase II, induces DNA damage and apoptosis
JQ1	none	DMSO	Bromodomain 4
Src inhibitor I	none	DMSO	Src, Lck, VEGFR2, c-fms
Bortezomib	Velcade	DMSO	Proteasome

3.2.d. Proliferation assay and data analysis

For the proliferation assay, DH82 and BD cell lines were seeded on 96-well plates with 7,500 cells/well and 12,000 cells/well, respectively. The cell cultures were then placed in the incubator at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Subsequently, the culture medium was replaced by 100 µl of medium with serial drug dilutions for 72 h. Control wells without cells were prepared containing either water, medium alone, and medium alone with the increasing drug concentrations.

The proliferation rate of the cells were measured using a CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI) after 72 h of incubation time. First, for each well, 20 µl of a solution containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], together with an electron coupling reagent phenazine methosulfate (PMS) was added at a ratio of 10:2, according to the manufacturer. The cell cultures were incubated for additional 2 hours and then the absorbance of the formazan product was measured using EnVision® Multimode Plate Reader (PerkinElmer, Waltham, MA) at a wavelength of 490 nm.

The background absorbance, due essentially to the reaction of the culture medium and each drug, was subtracted from the absorbance values generated by the cells exposed to drugs. The effect of cell proliferation by each drug was calculated as follows: proliferation (%) = [1-(A-B)/(C-B)] x 100 where A is the response with compound, B is the background response with no drug, and C is the response with vehicle (1% DMSO). The absorbance generated by the “cells alone” control is denoted as $AbsIC_{100}$ (100%) and the absorbance generated by water control is denoted as $AbsIC_0$ (0%). The

calculated cell proliferation percentage of cells at each (log10) drug concentration was then plotted using GraphPad Prism 5 software nonlinear regression curve fitting.(PRISM 5, GraphPad Software, La Jolla, CA, USA). IC_{50} is defined as the concentration of the drug that inhibits cell growth by 50%, and it was determined as the corresponding drug concentration value of the mean between 0 and 100% proliferation rate.

3.3. RESULTS

3.3.a. Inhibitory effects of drugs on HS cells proliferation

Dose-response curves were generated with the results from the MTS proliferation assay; from which the respective IC_{50} values were determined **(Figure 3.1)**. The response was variable between the different drugs, and also between the two cell lines. When present, the inhibitory effect on HS cells growth was in a dose-dependent manner. The results from IC_{50} **(Table 3.2)** values indicated that dasatinib, doxorubicin, JQ1 and bortezomib were the compounds that elicited an effective inhibitory response within or close to a pharmacologically relevant concentration (<100nM). While dasatinib and doxorubicin were effective against BD cell line, and not against DH82; JQ1 was effective against only DH82; and bortezomib showed efficacy against both HS cell lines.

Table 3.2: Results of IC_{50} (nM) values from drugs used on two HS cell lines

List of the drugs IC_{50} (nM) values for each HS cell line		
Inhibitor	BD	DH82
Dasatinib	**44**	3,340
Erlotinib	3,226	4,383
Gefitinib	30,730	46,410
Imatinib	34,020	39,210
Masitinib	15,780	39,150
Nilotinib	29,920	26,230
Toceranib	1,901	1,738
Sorafenib	36,350	13,660
Sunitinib	4,560	17,950
Tozasertib	7,740	1,370
CCNU	105,300	139,500
Cladribine	64,030	222,000
Doxorubicin	**130**	320
JQ1	260	**110**
Src inhibitor I	36,150	> 10,000
Bortezomib	**30**	**10**

Figure 3.1: Results of dose-response curves of both cell lines, BD (•) and DH82 (■), generated from the drug-screening assay using the following 16 drugs: (A) dasatinib, (B) erlotinib, (C) gefitinib, (D) imatinib, (E) masitinib, (F) nilotinib, (G) toceranib, (H) sorafenib, (I) sunitinib, (J) tozasertib, (K) lomustine, (L) cladribine, (M) doxorubicin, (N) JQ1, (O) src inhibitor, (P) bortezomib

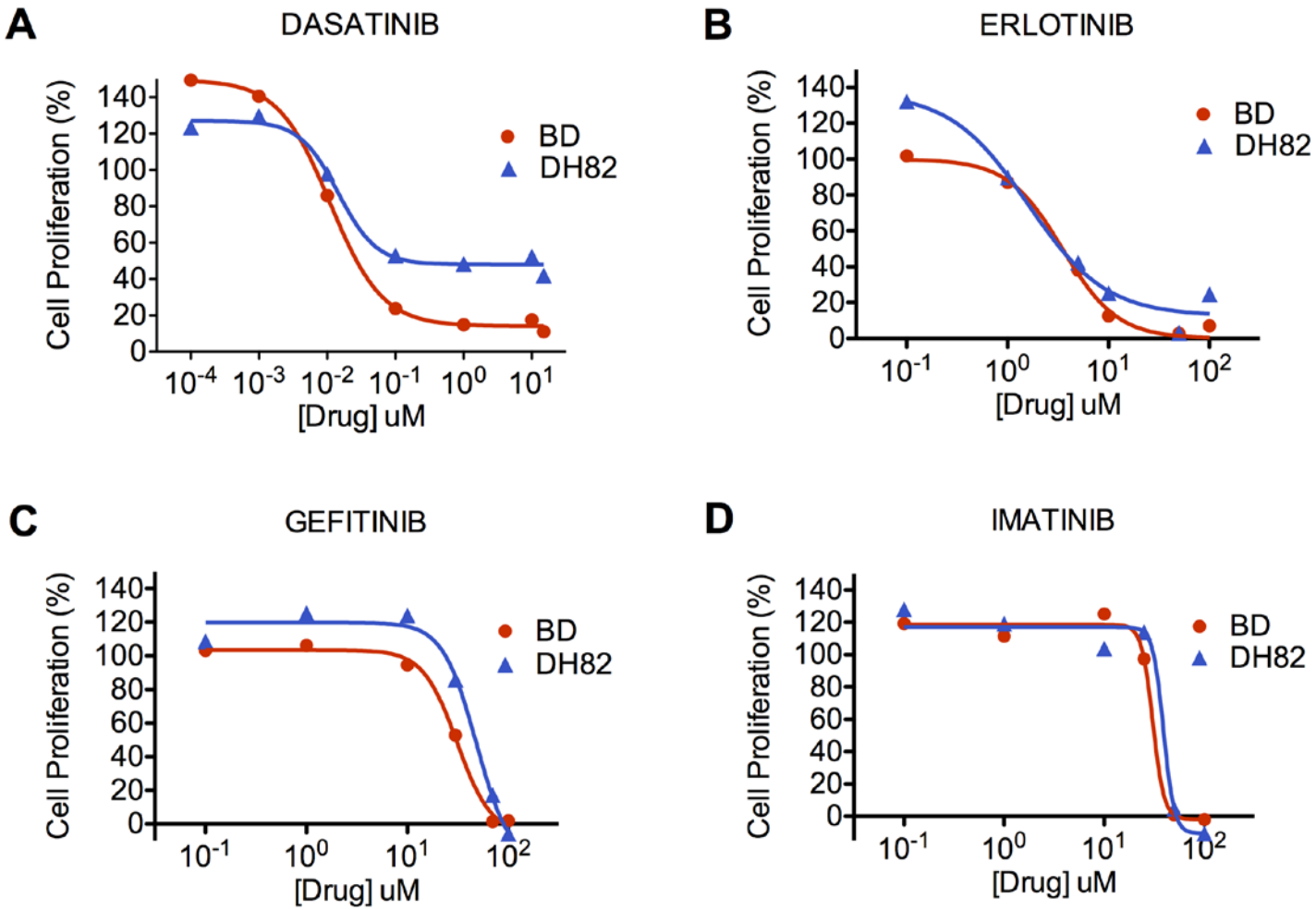


Figure 3.1 (cont'd)

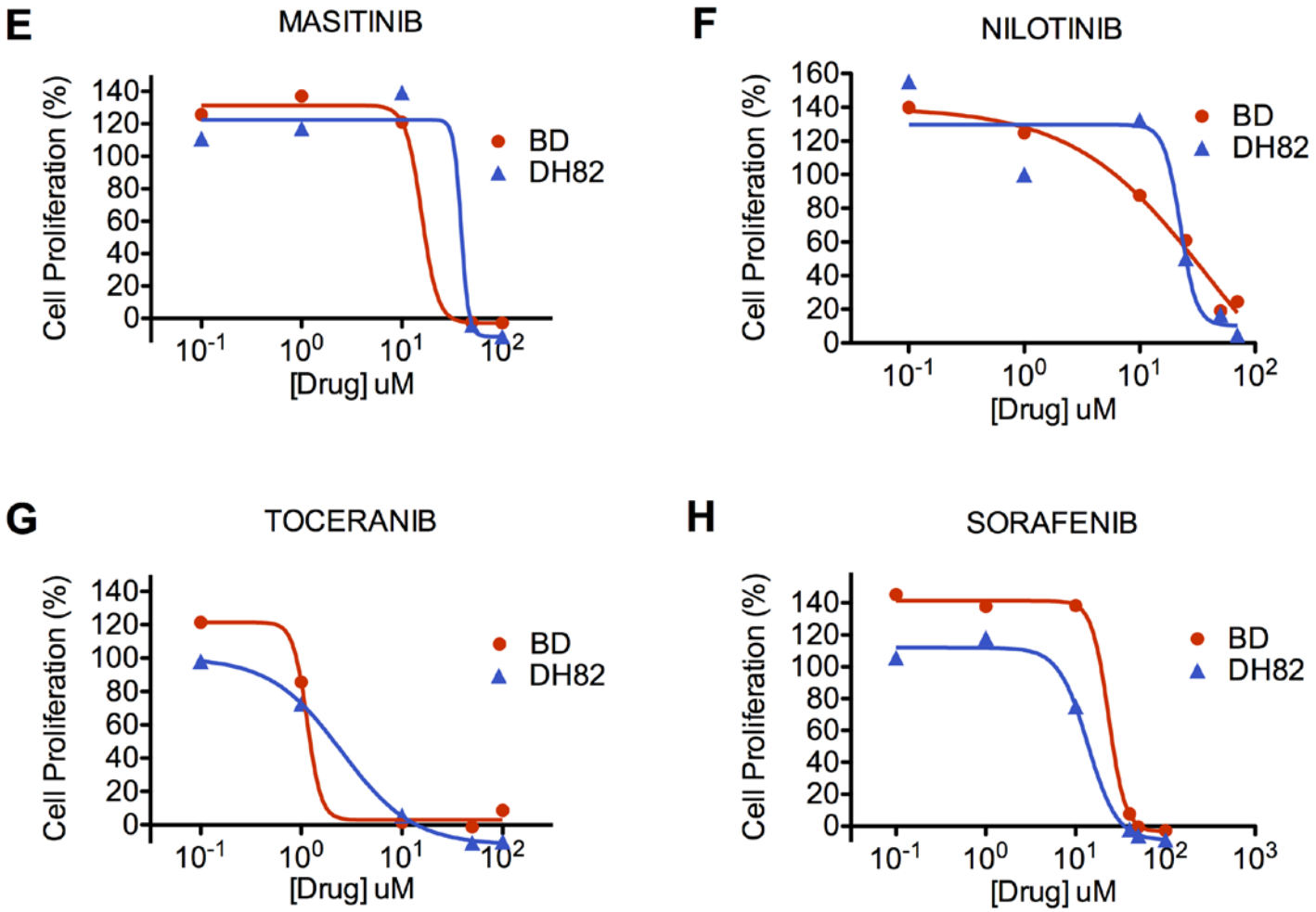


Figure 3.1 (cont'd)

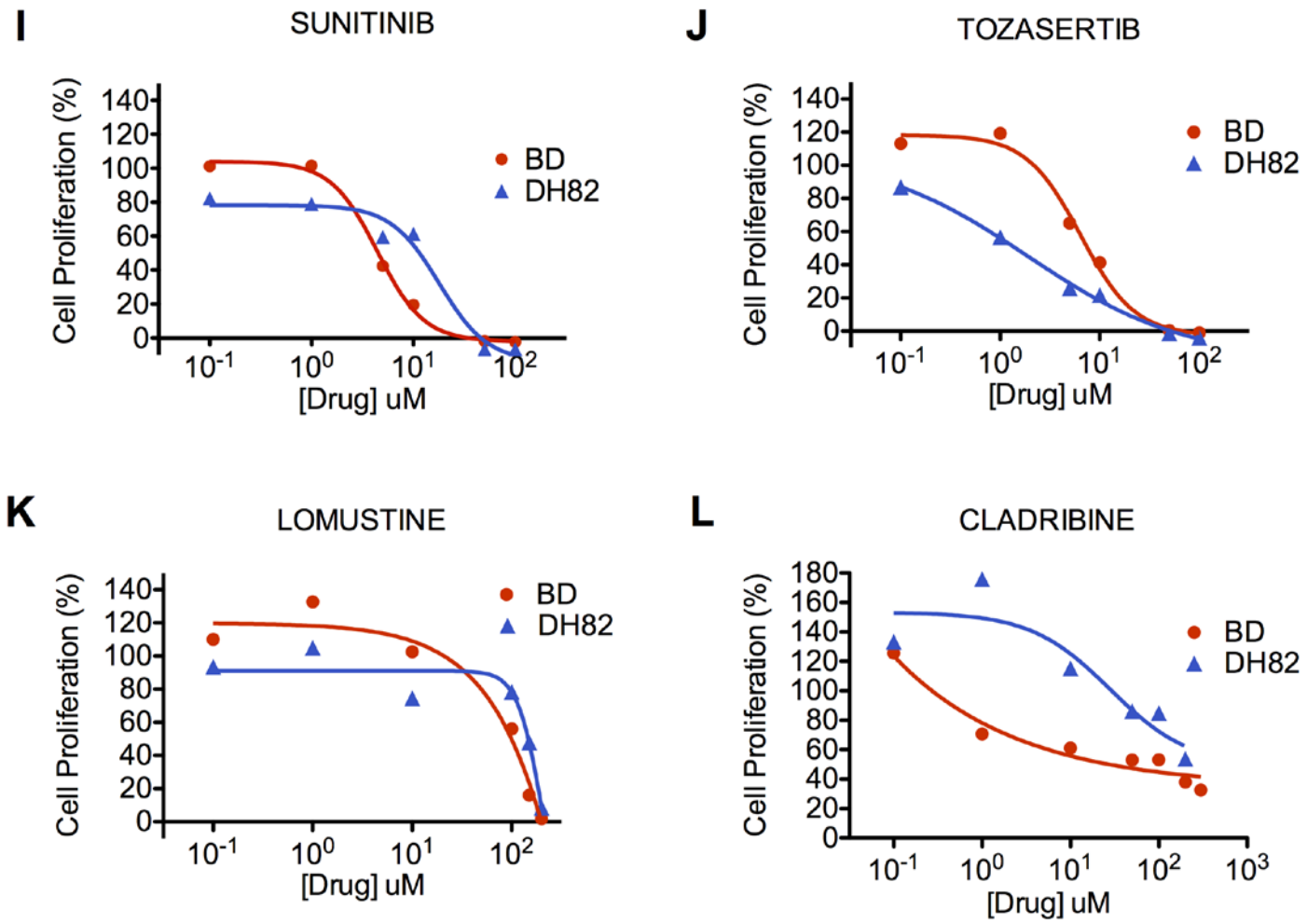
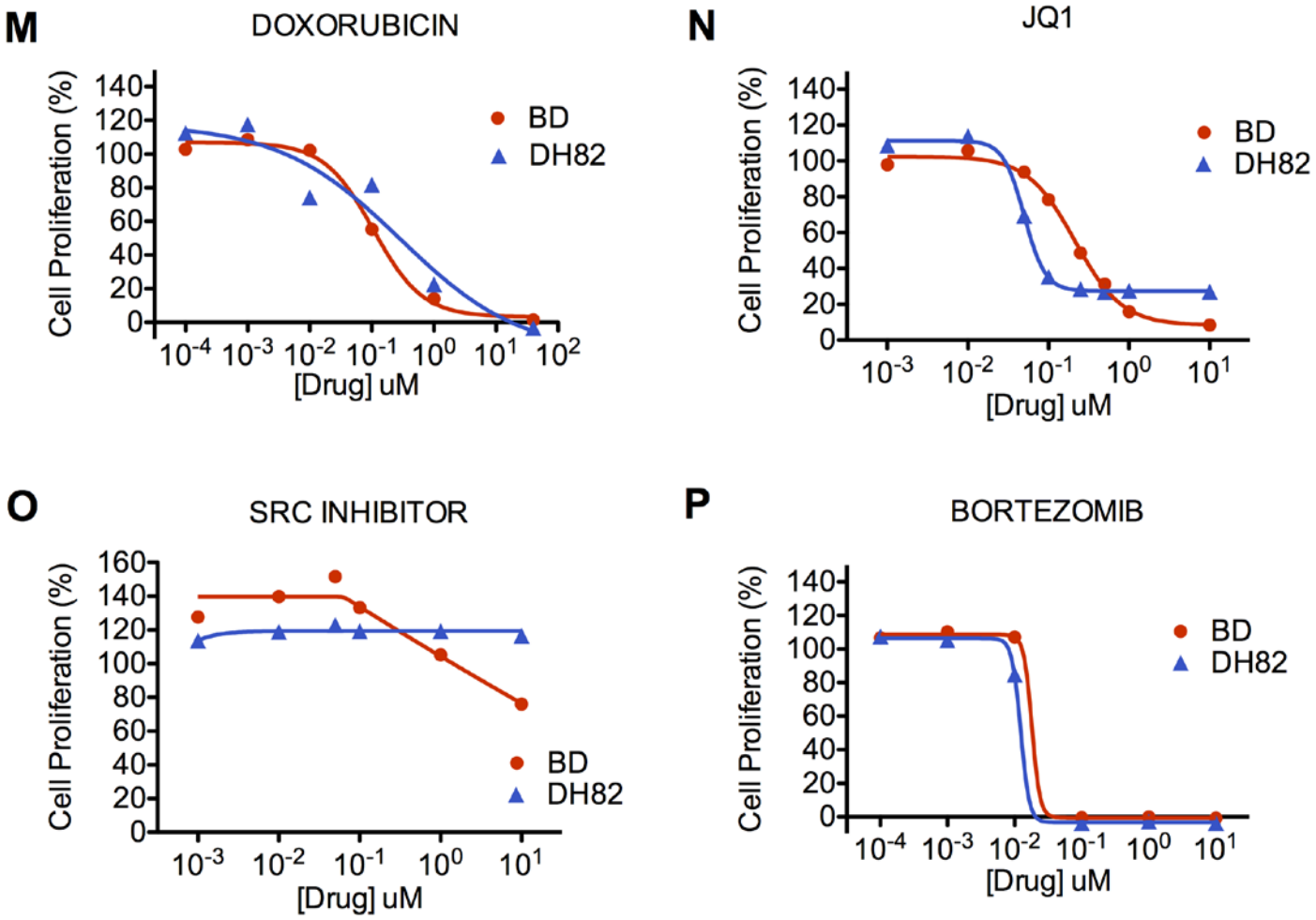


Figure 3.1 (cont'd)



3.3.b. Assessing feasibility of a panel of TKIs using maximum achievable plasma concentrations

The maximum achievable plasma concentrations of the drugs were based on the plasma concentration values on the no-observed-adverse-effect level (NOAEL), or on the therapeutic plasma concentration described in the veterinary and human medicine literature. When available, the plasma concentration values encountered for dogs were used over those for humans (**Table 3.3**). Due to the fact that the bromodomain inhibitor drug JQ1 use has been limited to studies with mice, the relevant plasma concentration was defined as the concentration known to be therapeutic experimentally in mice. The use of the src inhibitor I drug has never been reported in vivo, so the reference value is related to the reported drug concentration necessary for the molecular target inhibition.

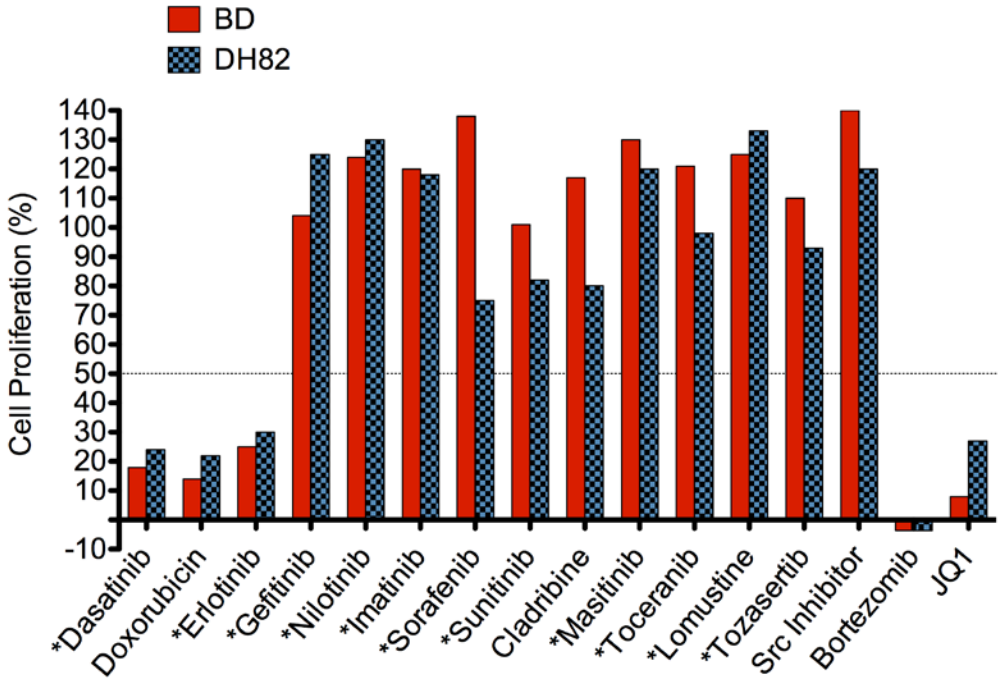
Next, the effect on cell proliferation rate at each drug maximum achievable plasma concentration was verified in both cell lines **(Figure 3.2)**. Only 5 drugs demonstrated to effectively inhibit cell proliferation at the maximum plasma concentration: dasatinib, doxorubicin, erlotinib, bortezomib and JQ1.

Table 3.3: List of achievable plasma concentration of drugs described in the veterinary and human medicine literature, and the corresponding cell proliferation rate

Drug	Specie	Dose	Effect	[plasma] (nM)	Proliferation (%)		Ref.
					BD	DH82	
Dasatinib	dog	3 mg/kg	NOAEL	300	18	24	[69]
Erlotinib	dog	150 mg	TD	6,978	25	30	[70]
Gefitinib	dog	100 mg/kg	NOAEL	2,662	104	125	[71]
Imatinib	dog	10 mg/kg	NOAEL	660	120	118	[72]
Masitinib	dog	10 mg/kg	TD	1,500	130	120	[63]
Nilotinib	dog	20 mg/kg	NOAEL	1,488	124	130	[73]
Toceranib	dog	3.25 mg/kg	TD	100	121	98	[31]
Sorafenib	human	400 mg	TD	13,338	138	75	[74]
Sunitinib	human	50 mg	TD	125	101	82	[75]
Tozasertib	human	8 mg/m^2	TD	274	117	80	[76]
CCNU	human	130 mg/m^2	TD	4,278	110	93	[77]
Cladribine *	human	0.09 mg/kg	TD	28	125	133	[78]
Doxorubicin *	dog	30 mg/m^2	TD	1,130	14	22	[79]
JQ1**	mice	50 mg/kg	TD	21,882	31	27	[80]
Src inhibitor	In vitro	-	Src inhibition	44	140	120	[81]
Bortezomib*	human	1.3 mg/m^2	TD	291	-3.6	-3.6	[82]

* Compounds administered intravenously
** Compound administered intra-peritoneally

Figure 3.2: Effect of drugs on the proliferation rate of HS cells at the maximum achievable plasma concentration

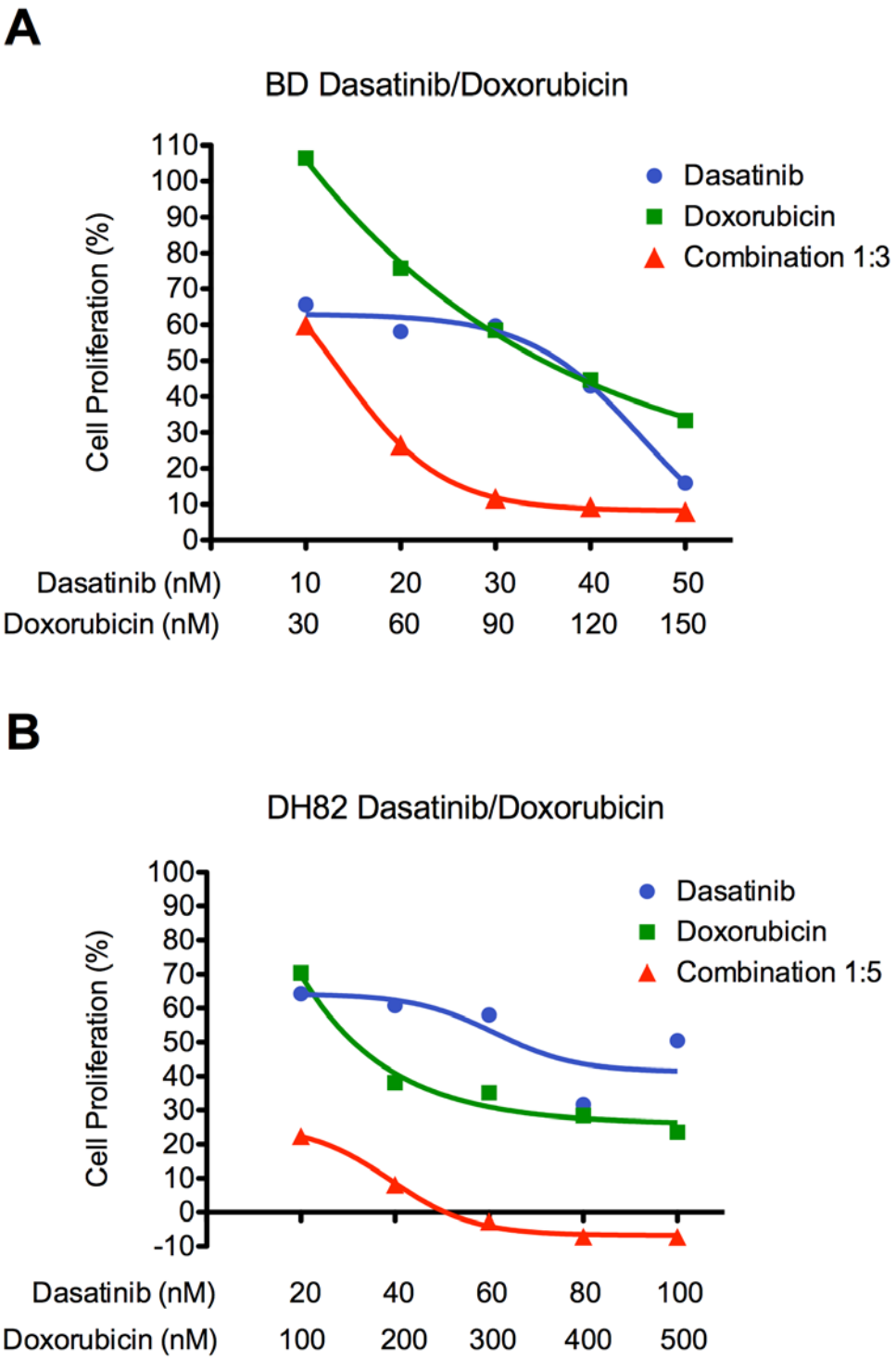


*Compounds that are administered by mouth to the patient

3.3.c. Exploring synergistic combinations

We next assessed the cytostatic effect caused by the combination of dasatinib and doxorubicin. Both cell lines were incubated with an increasing series of 5 concentration of each drug together or separately. Drug concentrations were defined in a way to bracket the IC_{50} for each drug and each cell line. Dasanitib and doxorubicin concentration ratios were 1:3 and 1:5 for BD and DH82 cell lines, respectively. Cell proliferation was measured by MTS proliferation assay. As observed in **Figure 3.3**, the drug combination produced clearly an additive antigrowth effect on both cell lines.

Figure 3.3: The inhibitory effect on cell proliferation of dasatinib and doxorubicin administered alone and in combination against BD (A) and DH82 (B) cell lines.



3.4. DISCUSSION

As a result of the better understanding of cancer biology, and the fundamental molecular pathways involved in oncogenesis, the focus of research in oncology has switched from conventional unspecific chemotherapeutic drugs to the development of molecularly-targeted compounds. The deregulation of molecules such as protein kinases are involved in several types of cancer, therefore are considered promising targets for therapeutic intervention.[25, 53, 83]

Toceranib and masitinib are examples of well-tolerated TKIs, that have demonstrated favorable antitumor responses against a wide variety of canine malignancies, including mast cell tumor, GIST and various carcinomas.[17, 19, 64] The positive clinical results and the versatile characteristic of TKIs, encouraged us to explore their therapeutic effect for canine HS, since to date, this malignancy carries a poor prognosis due to the lack of effective treatment.

In order to search for drug candidates for the treatment of dogs with HS, we conducted an *in-vitro* drug-screening experiment using a collection of 16 drugs in 2 HS cell lines, as previously described. Results from our study showed that the response was variable between the different drugs, and across the different cell lines.

The variability of response exhibited between different cell lines to a given inhibitor was expected. It reflects the tumor genetic heterogeneity associated with the existence of not one, but multiple critical pathways involved in oncogenesis.[24] In the present study, BD is a novel HS cell line established over the past 2 years in our laboratory, in the other hand, DH82 is a commercially available cell line that has been established in 1988, and so it has been submitted to numerous cell passages, increasing the chances

of new genetic mutations, that possibly resulted in a modified genetic structure in comparison to a novel primary cell line. [84] Furthermore, BD is derived from a BMD and DH82 was derived from a Golden Retriever.

In order to determine the effective drugs, we searched for those with lower values of IC_{50}. Values of IC_{50} around 100 nM were considered as pharmacologically relevant. From a total of 16 compounds, dasatinib, doxorubicin, JQ1 and bortezomib effectively inhibited the cell growth of at least one HS cell line.

Dasatinib is considered a tyrosine kinase pan-inhibitor because it has multiple main targets, including the Src family kinases (Src, Lck, Yes, and Fyn), the Bcr-Abl, and also c-KIT, PDGFRβ and EphA2 in a lower magnitude.[85] Dasatinib has been applied as first line therapy for CML in chronic phase in humans patients, with favorable long-term response comparable to its antecedent TKI imatinib.[86, 87] Its use *in vivo* has never been documented in veterinary oncology, but interestingly, dasatinib was also considered effective against 4 out of 7 canine HS cell lines in a study from a Japanese group published while this study was in progress. The calculated IC_{50} values from that study varied from 5.4 to 54.5 nM, similarly to the IC_{50} value of 44 nM found in the present study.[24]

Drug-screening assays with molecularly-targeted drugs may provide, in theory, the opportunity to better understand the signaling pathways driving tumorigenesis. However, most of the protein kinase inhibitors target multiple kinases simultaneously, therefore, the identification of the important driver molecules by this method can be quite challenging. In the present study, the fact that dasatinib is known to inhibit src

kinase at extremely low concentrations (4.1 nM), could lead to the hypothesis that src signaling is important for HS development in dogs.[88] However, the src inhibitor showed minimal effect against the canine HS cell lines **(Figure 3.1.O)**, which means that the antitumor activity of dasatinib was through mechanisms other than src kinase inhibition. Other studies have reported that src kinase inhibition was not the main mechanism of action of dasatinib against various types of tumors *in vitro* (human hepatocellular carcinoma, head and neck squamous cell carcinoma (HNSSC), and thyroid cancer).[89-91] Another interesting evidence of the broad-spectrum of this drug was reported in an activity-based kinase profiling study where dasatinib had some activity against more than 200 kinases at 1 μM in a panel of 310 human kinases, and based on the IC_{50}, dasatinib had 38 main target kinases.[88]

This versatility observed in most of TKIs may also explain the discrepancy behind the fact that, although dasatinib and other TKIs have common molecular targets, they were not as potent as dasatinib against the HS cells. An alternative explanation could be that even sharing specificity for the right target, other TKIs have lower degrees of affinity.

In the present study, doxorubicin was the only conventional chemotherapeutic drug that elicited a favorable response against the HS cell lines. Doxorubicin is an anthracycline antibiotic drug widely used in veterinary oncology to treat diseases such as canine lymphoma, soft tissue sarcoma, and osteosarcoma.[92-95] Its antitumor mechanisms involve the intercalation of DNA, inhibiting protein synthesis and eliciting the formation of free radicals, and inhibition of topoisomerase II. [96] Our results agree with previous studies that considered doxorubicin an effective drug against canine HS

both *in vitro* and *in vivo*. [8, 44] In the clinical study, doxorubicin had similar response rates (46%) to those treated with the standard of care chemotherapy drug CCNU.[8]

The combination of dasatinib and doxorubicin resulted in at least a favorable additive effect **(Figure 3.3)** against the HS cells. Synergistic activity *in vitro* between TKIs and cytotoxic drugs was also observed in previous studies.[97, 98] And when dogs with MCT were treated with the combination of the TKI toceranib and vinblastine, a conventional chemotherapeutic drug, the dose of vinblastine had to be reduced to be well-tolerated. However, the combination of these drugs seems to promote additive or synergistic anticancer effect.[22]. The combination of drugs with different mechanisms of action should be considered as a relevant strategy to optimize the therapeutic effect of each inhibitor.

In the present study we tested the novel molecularly-targeted drugs JQ1 and bortezomib. Surprisingly, both drugs demonstrated to have a favorable inhibitory activity against the growth of HS cells.

JQ1 (thieno-triazolo-1,4-diazepine) is a novel bromodomain and extraterminal (BET) inhibitor. One mechanism of action is by suppressing the BRD4 member of this subfamily of bromodomain proteins. BRD4 functions as a transcriptional regulator, binding to MYC promoter region and facilitating transcriptional activation.[99]. JQ1 binds competitively to acetyl-lysine recognition motifs, or bromodomains, displacing BRD4 fusion oncoprotein from chromatin, and therefore inhibiting c-Myc-dependent transcription.[80, 100] In normal cell, the expression of *c-myc* gene is tightly controlled and regulated. While resting cells express low level of this gene, *c-myc* expression increases with the stimulation from external signals such as growth factors, extracellular

matrix contacts, and cell cycle signaling. In cancer cells, the activation of this oncogene has been described to happen through several mechanisms: provirus integration, gene amplification, and chromosomal translocation.[101] The expression of *c-myc* was explored in only a few types of cancer in dogs, in the other hand *c-myc* overexpression is critical for tumorigenesis in a wide variety of human cancers, including pediatric neuroblastoma, breast carcinoma, and small-cell lung carcinoma.[102-104]

Although the use JQ1 is still limited for research on *in vitro* and in mice models, it showed promising results on the treatment of hematologic malignancies such as human multiple myeloma, lymphoma, and acute myeloid leukemia.[80, 105, 106] This is the first study reporting the use of JQ1 *in vitro* as anticancer agent for dogs, and it represents evidence of potential drug candidate for the treatment of HS in dogs.

The second effective novel molecular-targeted drug used in this study was bortezomib. Bortezomib (*N*-pyrazinecarbonyl-*L*-phenylalanine-*L*-leucine boronic acid) is a reversible specific inhibitor of the proteasome pathway, which inhibits the growth of cancer cells by inducing apoptosis and overcomes resistance to other drugs by inhibiting NF-κB activation.[107]

The proteasome is a multi-catalytic enzyme complex present in all eukaryotic cells, and has a major role in processing and degrading proteins, many of which are essential for cell proliferation signaling and cell cycle regulation, as well as apoptosis.[108] Examples include the tumor suppressor protein p53, various cyclins, cyclin-dependent kinase inhibitors p27 and p21, and the NF-κB inhibitory protein IκB.[109] Bortezomib was approved by U.S. FDA for the treatment of human multiple myeloma and mantle cell lymphoma, although its use has been expanded to several other types of cancer

alone, or in combination with other drugs.[110-113] The same mechanism of action from bortezomib was observed in an experiment with healthy dogs that showed proteasome inhibition at doses below the established MTD, indicating that the effective dose for dogs might be well-tolerated.[114] The growth of canine lymphoma cell lines was inhibited *in vitro* by bortezomib at low IC_{50} values ranging from 1.24 to 5.56 nM, which was associated to the inhibition of the canonical NF-κB pathway.[115] In the present study, bortezomib also had remarkable low IC_{50}s of 30 nM and 10 nM for Bella Donna and DH82 HS cell lines, respectively, which indicates that the proteasome can be a potential therapeutic target of canine histiocytic sarcoma.

After determining that the effective drugs against the HS cell lines were dasatinib, doxorubicin, bortezomib and JQ1, we verified if these drugs would still be effective at the maximum achievable plasma concentration described in the literature. The effect caused by a drug is related to the concentration of the drug at the receptor site. The concept of a receptor-drug interaction model is that the drug concentration at the receptor site is in equilibrium with the drug concentration in the plasma (one compartment model).[116] This assumption lead us to relate drug action to plasma concentration, and therefore, to the drug concentration in the medium of a cell culture. If the IC_{50} values determined *in vitro* are equal or below the values of maximum achievable plasma concentration determined *in vivo*, it suggests that these drugs are effective in concentrations that are feasible and tolerable for the patient. Dasatinib, doxorubicin, bortezomib and JQ1, all had IC_{50} values below the maximum achievable plasma concentration. Moreover, at the maximum achievable plasma concentration, all

these drugs inhibited the HS cells growth in more than 60%.

The present study was able to produce results that raise relevant questions regarding the treatment of histiocytic sarcoma in dogs using molecularly-targeted drugs and the critical signaling pathways affected by these drugs, which confer their therapeutic antitumor activity. However, this study has a few limitations to be address.

Firstly, the data obtained in this study is a result of single experiment sets. Ideally each proliferation assay should be performed in at least triplicate, so we could confirm the consistency of the results.

Secondly, the use of data produced from *in vitro* studies is should be considered preliminary, because cancer cell cultures are far from mimicking the *in vivo* microenvironment, which are involved on the tumor growth, survival, and dissemination. Factors such as the presence of immune cells and their cytokines, factors generated from the extracellular matrix, blood circulation, or hypoxic environment are excluded from *in vitro* assays. However, the goal of *in vitro* studies is to select drugs with a high potential to be tested *in vivo*, or in a clinical setting of investigation. *In vitro* studies may optimize time of research, avoid extra costs, and ensure a low risk/benefit ratio for the patient.[117] One study actually could show a significant relationship between cytotoxicity of chemotherapeutic drugs for cancer cells *in vitro,* and achievable systemic exposure in humans, suggesting that *in vitro* assays may provide useful information in the development of anticancer drugs.[118]

Lastly, another limitation on the use of *in vitro* assays is the lack of the several pharmacokinetics factors present in living organisms that may alter the efficacy of a drug. As an example, in the present study, pure compounds from the manufacturer or

from research laboratories were used, however, most of the drugs are administered by mouth to the patient, therefore they are subjected to first-pass liver metabolism. Other factors include *in vivo* drug instability, plasma protein binding, low bioavailability, competitive pathways, slow excretion, and other pharmacokinetic factors.[119] However, the fact that several drugs, alone and in combination could inhibit the growth of the tumor cells at concentrations below the maximum achievable plasma levels is highly encouraging and justify further *in vivo* studies.

In vitro studies are practical and useful methods to determine the potential therapeutic effect of a receptor-targeting compound before proceeding to experiments using animal models or patients. Further evaluations *in vivo* are needed to build on the results obtained in the present study to provide effective treatment against this disease in dogs and for similar diseases in humans.

CHAPTER 4

Conclusions and Future Directions

The results from the present study suggest that molecularly-targeted compounds are part of a promising anticancer therapeutic approach in dogs, especially for tumors such as HS that possess an aggressive behavior and that manifest a limited short-term response to conventional cytotoxic chemotherapeutic dugs.

The tyrosine kinase pan-inhibitor dasatinib effectively inhibited HS cells growth *in vitro*. The antitumor activity produced by dasatinib was augmented when combined to doxorubicin, a conventional chemotherapeutic agent, suggesting that clinical responses may be improved using both drugs together. Novel molecularly-targeted drugs also showed encouraging antitumor activity against HS cells, including the BET bromodomain inhibitor JQ1, and the proteasome inhibitor bortezomib.

These compounds showed low values of IC_{50}s for one or more HS cell lines *in vitro*. To further evaluate the therapeutic implications of these drugs in canine HS, studies *in vivo* using xenograft murine models should be pursued. Animal models are useful in confirming the potential antitumor effect observed *in vitro*, and also in exploring drug-related toxicity, so follow clinical trial settings investigations can be pursued.

Anticancer drug screening experiments using molecularly-targeted drugs offer the possibility to elucidate the critical signaling pathway driving the malignant transformation. The molecular targets of dasatinib, JQ1 and bortezomib are well characterized, and so they can be further explored. One approach is look at the expression of these proteins through an immunohistochemical analysis from a canine

histiocytic sarcoma formalin-fixed, paraffin-embedded tissue microarray using antibodies specific for those target molecules of interest, including c-myc, PDGFR, c-KIT, SRC-family, EPHR, and EGFR. Recent studies have shown an alternative approach that involves the use of siRNAs targeting the genes from those proteins, followed by evaluation of the effects on cell viability.[25, 120]

The results from the present study suggest that molecularly-targeted therapy is a promising modality of treatment for dogs with HS, and further studies are needed to confirm the potential of these drugs, and also to explore the role of the related pathways. Such studies can pave the way for better treatments for not only dogs with HS, but also for humans with similar disorders.

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