

Inhibition of Cell Signalling in Neoplastic Canine Mast Cells

By

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ABSTRACT

Inhibition of cell signalling in neoplastic canine mast cells

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Canine mast cell tumours (MCTs) are a common skin malignancy in dogs, largely driven by dysregulated activity of KIT and VEGFR2. High grade/metastatic MCTs are resistant to conventional chemotherapeutics, thus canine MCT cells represent a valuable model for KIT dysregulation in human cancers. Canine MCTs are currently treated with the TKIs toceranib (Palladia) or masitinib (Kinavet), with mixed results. Itraconazole, an anti-fungal agent shown to interfere with VEGFR2 glycosylation and hence signalling, may also affect KIT in these cells. The effect of itraconazole on two canine MCT cell lines (MCT1 and MCT2) was evaluated individually and in combination with RTK inhibitors. Cell proliferation and receptor phosphorylation and glycosylation were examined after treatment. Combination itraconazole and toceranib treatment for 48 hours generally decreased proliferation rates and disrupted RTK phosphorylation and glycosylation. These data demonstrate that novel anti-cancer drugs such as itraconazole warrant further investigation as inexpensive and less toxic alternatives to traditional chemotherapeutics.

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DECLARATION OF WORK PERFORMED

I declare that all work reported in this thesis was performed by me.

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LIST OF ABBREVIATIONS

14DM	Lanosterol 14 α -demethylase
ATP	Adenosine 5' triphosphate
BAEC	Bovine aortic endothelial cell
BSA	Bovine serum albumin
CD117	KIT; Cluster differentiation 117; receptor tyrosine kinase
<i>c-KIT</i>	Gene encoding KIT (CD117), the stem cell factor receptor
CML	chronic myelogenous leukemia
DAPI	4',6-Diamidino-2-Phenylindole
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
Erk	extracellular signal-regulated kinases
FBS	Fetal bovine serum
GIST	Gastrointestinal stromal tumours
HUVEC	Human umbilical vein endothelial cell
IF	Immunofluorescence
IGF-IR	Insulin-like growth factor receptor
JAK-STAT	Janus kinase- signal transducers and activators of transcription
JM	Juxtamembrane
kDa	Kilodalton
Ki67	Protein encoded by MKI67 gene; immunohistochemistry with monoclonal antibody MIB-1 detects Ki67 to assess cellular growth fraction
KIT	CD117; receptor tyrosine kinase; stem cell factor receptor
MCP-1	Monocyte chemoattractant protein-1
MCT	Mast cell tumour
mTOR	Mammalian target of rapamycin, also known as atypical protein kinase
NSCLC	non-small cell lung cancer
p53	Protein 53
PBS	Phosphate buffered saline
PGE-2	Prostaglandin E2
PI3K	Phosphoinositide 3'-kinase
PLC γ	Phospholipase C gamma
PMSF	Phenylmethylsulfonyl fluoride
PTM	Post-translational modification
PVDF	Polyvinylidene fluoride
rER	rough endoplasmic reticulum
RTK	Receptor tyrosine kinase
SCLC	Small-cell lung cancer
SDS	Sodium dodecyl sulfate
SFK	Src family kinase
SH2	Src homology 2
SHB	SH2 domain-containing adaptor protein B
TBS	Tris-buffered saline
TBS-T	Tris buffered saline - tween 20
TEMED	N,N,N,N-Tetramethylethylene diamine

TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor (-1,-2,-3)
VRAP	VEGFR-associated protein
WCL	Whole-cell lysate
WHO	World Health Organization

INTRODUCTION

Mast cells are immune system cells present in most tissues and organs of the body, with higher concentrations at the body-environment interface [1]. These cells contain cytoplasmic granules loaded with inflammatory mediators, proteases, cytokines and histamine [2, 3]. Through the release of histamines, mast cells play a critical role in the IgE and IgG mediated allergic responses [4]. Despite their importance for normal physiological processes, mast cells can be prone towards neoplastic transformation [5, 6]. This leads to the development of mast cell tumours (MCTs) in many animals, especially canines [5, 6]. A sole underlying cause of transformation has yet to be identified, though dysregulated receptor tyrosine kinase (RTK) activity is highly implicated [7-9].

RTKs are growth factor receptors that regulate normal cellular function by phosphorylating a multitude of proteins and substrates [10, 11]. The mode of action generally involves binding of ATP to the binding pocket [12]. Phosphate groups are then transferred to tyrosine residues on the receptor's cytoplasmic tail or on other molecules [12]. This results in the activation of intracellular signalling pathways that ultimately alter levels of gene transcription [10-12]. RTKs have been identified in cell growth, survival and differentiation pathways [9, 12-14]. Dysregulation of RTKs is therefore a critical step in the development of many cancers, including MCTs [13, 15, 16].

MCTs account for 7-21% of all canine skin cancers, making this disorder the most prevalent skin neoplasm in dogs [17-19]. The tumours arise in the dermis or subcutaneous tissue, and have highly variable clinical presentations and prognoses [20, 21]. Current grading schemes developed decades ago by Patnaik *et al.*, (1984) have proven useful, if flawed. Tumours are classified as grade I (low risk), grade II (intermediate risk) and grade III (high risk) [22]. While

these guidelines have been helpful to pathologists and veterinarians, MCTs are often misclassified based on subjective diagnoses [22]. To further complicate MCT treatment, high risk and metastatic cases are often highly resistant to traditional chemotherapy drugs [23]. Combined surgery and aggressive chemotherapy is often ineffective, and survival times rarely extend past 12 months [24-27].

Poor treatment response has led to the examination of alternative strategies, such as RTK targeting [12, 28, 29]. RTK inhibitors, or tyrosine kinase inhibitors (TKI), are small molecule inhibitors that typically target the ATP binding pocket to block activation of the receptor [30, 31]. TKIs have been widely used in humans, and have been shown to be more specific and less toxic than standard chemotherapeutics [12, 28]. Toseranib (Palladia), masitinib (Kinavet) and imatinib (Gleevec) have all been successfully employed in dogs to treat various diseases [12, 32]. However, TKIs have several significant drawbacks. TKIs are often exorbitantly expensive, due to the costly and time consuming process of developing small molecule inhibitors [12]. This is a key consideration for veterinary oncology, since euthanasia is often the only viable option for pet owners faced with costly treatment plans. TKIs also exhibit highly variable efficacy, especially when treating high grade or metastatic disease [33]. Finally, inherent or developed drug resistance often renders TKIs useless as a cancer treatment [34, 35]. Researchers have consequently focused on identifying pre-existing drugs that have novel anti-cancer effects. This process largely focuses on mass screening of FDA-approved drugs to determine any inhibitory effects on angiogenic mediators or cancer cell lines [36-38].

One drug that has been repeatedly shown to have antiangiogenic effects is itraconazole, a safe and widely used antifungal drug [39]. This drug has recently been found to interfere with VEGFR2 trafficking, glycosylation and signalling activity [39]. VEGFR2 is an RTK involved in

angiogenesis, the process of blood vessel formation from pre-existing blood vessels [40].

Itraconazole therefore exerts potent anti-angiogenic activity both *in vitro* and in mouse models, and has consequently been entered into numerous clinical trials as a new anti-cancer drug [39].

With this newly discovered information about novel anti-cancer drugs, I sought to determine whether itraconazole, solely and in combination with other TKIs, would disrupt the proliferation of canine MCT cell lines (MCT1 and MCT2) *in vitro*. In particular, I focused on the effects of itraconazole on post-translational modifications of two RTKs that are critical for mast cell proliferation and survival: KIT and VEGFR2.

CHAPTER 1 – LITERATURE REVIEW

MAST CELLS

Biology and Function

Mast cells were first characterized in 1877 by Paul Ehrlich, who incorrectly hypothesized that the cells were responsible for nourishing surrounding mesentery tissue [41]. This assumption was based on the distinctive staining pattern of abundant cytoplasmic granules [41].

Contemporary knowledge classifies mast cells as immune system cells derived from CD34+ hematopoietic progenitor cells in bone marrow [42]. Mast cells are relatively long lived immune system cells that take up residence in most tissues in the body. The highest concentrations of mast cells are located at the body-environment interface, such as the skin and mucosal linings [43]. Mast cells leave bone marrow in an undifferentiated state, and migrate to resident tissues to undergo a process called terminal differentiation [44]. A critical component of mast cell differentiation and survival is stem cell factor (SCF), the ligand for the KIT receptor [45]. SCF is largely supplied by fibroblasts and endothelial cells in the surrounding tissue matrix *in vivo* and co-cultures of fibroblasts *in vitro*. This ligand exists in soluble or membrane bound forms, and ligand binding to the KIT receptor contributes to migration, proliferation and survival pathways [46, 47].

Modern research has also revealed that mast cells are crucial for both innate and adaptive immune responses, primarily due to the contents of the cytoplasmic granules [1, 4]. These granules contain cytokines, proteases, chemotactic factors and histamines, making mast cells critical mediators of inflammatory and allergic responses [48-52]. Degranulation, the process of releasing intracellular granular components, can be mediated by physical, chemical, or immunological means. Heat, trauma and toxins are known inducers of degranulation [53]. The

primary mechanism of degranulation is antigen-specific IgE binding to IgE receptors, which are abundantly expressed on the mast cell surface [4, 54]. Antigen binding causes receptor crosslinking and activation of downstream signalling intermediates, which ultimately triggers degranulation. The release of cytokines and chemokines also assist with immune cell recruitment to sites of infection or damage [53].

Mast cells play a critical role in innate immunity via release of granular mediators [55, 56]. Their location in skin and mucosal areas provides strategic access to the front line defence areas of the body. This allows mast cells to act as sentinels to constantly monitor pathogen access points. If specific danger stimuli are detected from pathogens, surrounding tissue or other immune cells, mast cells are ideally placed to discharge their complement of cytoplasmic granules. In particular, release of histamine causes vasodilation and increased permeability of blood vessels [55, 56]. This leakiness enhances blood flow to damaged or infected areas, flooding the site with immune agents. Histamine can also cause increases in the activity of mucus producing endothelial cells and smooth muscle cells [55]. This initiates a dual defense response whereby increased mucus levels trap and inactivate pathogens, and muscle action expels the pathogens from the body. Mast cells are also capable of neutralizing toxins and directly killing pathogens via phagocytosis or the release of reactive oxygen species [57]. Secretion of anti-microbial peptides, such as cathelicidins, can also assist with defence against bacterial attack [58]. In addition, cytokines and chemokines create chemotactic gradients within the body, directing the recruitment of numerous immune and inflammatory cells towards sites of interest [56]. This could include eosinophils, basophils, natural killer cells and neutrophils [50]. Chemotactic gradients could also recruit antigen presenting cells, such as dendritic cells, making mast cells important for adaptive immunity [59]. Furthermore, studies show that mast cell-

deficient mice have impaired T-cell activation, indicating mast cells may be critical for T-cell adaptive responses [60, 61].

Mast Cells in Disease

Mast cells are involved in a number of human diseases. Uncontrolled growth of mast cells can lead to cutaneous mastocytomas (usually diagnosed in children) or systemic mastocytosis (usually diagnosed in adults) [62]. Both disorders are quite rare, with no worldwide epidemiological data available [62]. Epidemiological studies conducted in Denmark illustrate the uncommon occurrence of mastocytosis. Researchers looking at all types of adult systemic mastocytosis found an overall incidence rate of 0.89 per 100,000 per year [63].

Mast-cell mediated inflammatory and allergic responses can also lead to chronic inflammation disorders (such as cardiac fibrosis), hypersensitivity to allergens (asthma) and autoimmune disorders (such as rheumatoid arthritis) [64-66]. Due to the copious production of angiogenesis factors such as VEGF, mast cells are also implicated in tumour progression [67]. While most mast cell disorders are rare in humans, it is important to study their role in cancer and disease to enhance current treatment strategies. The most appropriate candidate for studying mast cells in disease is canines. Dogs make excellent models for human mast cell disorders due to large populations, higher incidence of mast cell disorders, and greater similarity to human disease progression than murine models [68, 69].

Canine Mast Cell Tumours

Canine mast cell tumours (MCTs) are common in dogs, representing 7-21% of all cases of canine skin tumours [21, 70]. The solid tumours arise primarily in the dermis and subcutaneous tissue, and initially present as a small raised nodule [21, 70]. While MCTs can

arise in any location on the body, the most common areas are the trunk (~50-60%), extremities (~40%), and the head and neck (~10%) [71-74]. Certain breeds are also predisposed to MCT development. For instance, the relative risk of MCT development is 16.7 and 8.0 times higher for boxers and Boston terriers, respectively [71]. These data seem to indicate an underlying genetic component to MCT development, however further research with larger sample sizes is needed to establish this [33]. The inconsistent nature of this neoplasm can make diagnosis difficult. MCTs can range from a small, benign node to large and ulcerated masses. MCTs additionally exhibit highly variable biological behaviour, from benign with good prognosis to aggressively metastatic [21]. The typical diagnostic process involves fine-needle aspirates and stained smears to examine the cytological appearance [19, 20]. Cytology and histological grading (see Table 1) is relatively successful at determining degree of differentiation and the clinical stage of the disease (see Table 2) [75, 76].

Treatment of Canine MCTs

Current treatment of canine MCTs is guided by the Patnaik grading scheme and the WHO stage classification seen in Table 1 and 2, respectively [20, 76]. Although the Patnaik grading scheme has historically been the gold standard guide for grading and treatment of canine MCT, the system has several drawbacks. Agreement among veterinary pathologists regarding MCT grading is variable, especially for distinguishing the often ambiguous differences between grade I and II tumours [77]. Relatively strict guidelines for grade III diagnoses can also lead to misclassification of some tumours likely to behave aggressively [77]. This leads to inconsistent MCT grading, which decreases the prognostic significance of the entire system. These limitations are important, since grading affects treatment plans, which in turn affect overall survival rates. Consequently, recent work by Kiupel *et al.* has attempted to rectify these

limitations by designing a two-tier grading scheme [77]. In this system, nuclear morphology and number of mitotic figures per 10 high-powered microscope fields determine whether a MCT is graded as high or low grade [77]. MCTs are classified as high grade if there is evidence of karyomegaly, or if there are at least 7 mitotic figures, 3 multinucleate cells or 3 bizarre nuclei per ten high-powered fields. All other cases are classified as low grade [77]. Using this system, Kiupel *et al.* found that agreement among pathologists increased significantly compared to the Patnaik system. When samples were graded under both systems, the high/low grade scheme also showed stronger correlation to mortality statistics than the Patnaik scheme [77]. This indicates that high/low grading offers more useful prognostic data for diagnosing and treating canine MCT. However, for the purposes of this work, focus will be placed on the established treatment protocols guided by Patnaik grading.

For grade I tumours, surgical resections are typically sufficient to cure the dog. Margins are generally recommended to be 2 cm laterally and at least 3 cm into the fascial plane, to remove the tumour tentacles that often extend deeper into subcutaneous tissue [33, 78]. The same often applies for grade II tumours, with some adjuvant treatment such as radiation or chemotherapy considered on a case-by-case basis. Grade III tumours typically require surgery, radiation, and systemic chemotherapy (usually vinblastine, lomustine, cyclophosphamide or prednisone) [26, 27, 33]. Median survival times for dogs with grade III tumours vary widely based on chemotherapy regimen and overall response of the tumour. One study by Hume *et al.*, 2011 reported a median survival time of 257 days for grade III cases after treatment with traditional chemotherapeutics [79]. More recent studies by Smrkovski *et al.*, 2013 reported median survival times of 630 days for dogs with grade II tumours [33]. This study was conducted using the TKI masitinib, which has proven safe and effective for use in canines [33,

80]. Caution should be used when interpreting these data however; since the sample size was relatively small, and the median survival times for dogs who did not respond (50%) were significantly lower (137 days) [33]. Despite these limitations, targeting of RTKs remains one of the most promising advances in treating canine MCTs [81].

RECEPTOR TYROSINE KINASES

Structure and Signalling

Kinases are enzymes that initiate the transfer of phosphate groups from adenosine triphosphate (ATP) to a substrate [15-17]. The phosphate group is typically transferred to a tyrosine, serine or threonine amino acid residue. RTKs are cell surface growth factor receptors that have several of these tyrosine kinase sites [15, 16]. As such, they act as phospho-transferases to either activate or inhibit a multitude of downstream targets. They are classified into different families based upon similarities in structural or signalling properties, but they all contain an extracellular ligand-binding domain, a transmembrane portion, a cytoplasmic kinase insert and a cytoplasmic terminal tail [10, 11, 31]. Two RTKs, KIT and VEGFR2, will be discussed in detail due to their significant role in mast cell proliferation and survival.

KIT Activity and Cellular Functions

The *v-kit* oncogene was first identified in 1986 as the gene responsible for the oncogenic properties of the Hardy-Zuckerman 4 feline sarcoma virus [82]. The cellular counterpart, (the proto-oncogene *c-kit*) and its protein product were then discovered one year later based on sequence similarities [83]. KIT, the protein product of *c-kit*, is a member of the Type III transmembrane RTK family [31, 84]. It is comprised of an extracellular region with five immunoglobulin-like domains (encoded by exons 1-9), a transmembrane domain (encoded by

exon 10), and an intracellular domain (encoded by exons 11-21) (See Figure 1 for schematic representation of KIT receptor) [84]. The intracellular domain is further divided into the negative regulatory juxtamembrane (JM) domain (encoded by exon 11), the kinase domain and the terminal tail (see Figure 1 for schematic representation of KIT receptor) [15-17, 85]. The *c-kit* sequence is highly conserved between dogs and humans, with 88% similarity, making canines a suitable model for studying human cancers driven by *c-kit* mutations [86]. Patients who suffer from disorders such as gastrointestinal stromal tumours (GIST) or acute myelogenous leukemia stand to benefit significantly from translational studies such as these [84]. The intracellular portion of kit consists of two major lobes, the large C-terminal lobe and the small N-terminal lobe. The catalytic kinase sites are located in the cleft between these two lobes [17, 87].

In the absence of ligand, KIT is autoinhibited by the JM domain. The JM domain inserts itself into the cleft, and sterically hinders movement of the two lobes [31, 87]. The Asp-Phe-Gly (DFG) motif in the beginning of the activation loop (A-loop) is in the DFG “out” conformation, with Phe811 protruding out and preventing ATP binding. This forces the receptor into a closed conformation [31, 87]. If the dimeric SCF ligand binds to two monomers of KIT, the normally monomeric KIT dimerizes. This brings homodimers of the tyrosine kinase residues into close proximity, leading to trans-phosphorylation of Tyr 568 and Tyr 570 on the JM domain [31, 87]. This kinase activity releases the JM from the cleft. The DFG motif moves to the “in” configuration and buries Phe811, permitting ATP to enter the binding pocket and initiate signalling pathways [31, 87].

The tyrosine phosphorylation sites act as docking points for a number of intracellular signalling intermediates. Docking of these intermediates initiates autophosphorylation and subsequent activation of several critical cellular pathways such as the PI3K, JAK-STAT, Ras-

Erk, SFK and Phospholipase C γ pathways [84] (See Figure 2 for schematic of KIT activation and associated pathways). The collective activation of these pathways is critical for a wide variety of cellular functions. These functions include, but are not limited to: DNA synthesis, cell survival, chemotaxis, protein/receptor/vesicular trafficking, cell division, cell cycle progression, adhesion, and membrane ruffling [84]. The importance of KIT in mast cells was illustrated by Kitamura and Hatanaka (1978), who showed that mice lacking functional KIT receptors had virtually non-existent populations of mast cells [88]. KIT activation by SCF is therefore critical for normal mast cell survival and function. Consequently, any disruption to KIT regulation or activity can have dire implications for the development of neoplastic disorders.

VEGFR-2 Activity and Cellular Functions

The vascular endothelial growth factor (VEGF) family regulates a wide variety of functions, including vascular development, lymphangiogenesis and angiogenesis [89]. VEGF-A was first functionally described by Senger *et al.*, (1983) as a vascular permeability agent that promoted leakage of plasma proteins to the extracellular matrix [90]. VEGF-A is a covalently linked homodimer and primary ligand for VEGFR-2, a RTK that has been extensively studied due to its role in angiogenesis [40, 89, 90]. Angiogenesis is the process of blood vessel formation from pre-existing blood vessel networks [40]. This process is typically quiescent in adult humans, except during wound healing and the female ovarian cycle. Angiogenesis involves the degradation of surrounding matrix tissue, the proliferation of endothelial cells and sprout formation into the matrix, ultimately creating functional new blood vessels [91]. Studies have shown that loss of one VEGF-A allele and VEGF-A $-/-$ is lethal in mice, clearly indicating their importance for normal vascular development [92, 93]. Although angiogenesis is extremely complex and involves numerous growth factors and receptors, VEGFR-2 and VEGF are believed

to be the primary inducers of endothelial cell proliferation [94]. This process is tightly regulated by a delicate balance of pro-angiogenic and inhibitory agents. An “angiogenic switch” occurs when the delicate balance is tipped in favour of the pro-angiogenic factors, initiating the angiogenic process [95, 96].

VEGFR-2 is a transmembrane, cell surface receptor belonging to the Type III family of RTKs. It is translated as a 150 kDa protein, and undergoes glycosylation to produce a mature 230 kDa form that is shuttled to the cell surface [97]. The extracellular domain consists of seven Ig-like domains (with domains II and III acting as the ligand binding domains [98, 99], a short transmembrane domain, and an intracellular tyrosine kinase domain with five primary phosphorylation sites (Y951, Y1054, Y1059, Y1175, Y1214) (See Figure 3 for VEGFR2 schematic) [100, 101]. Y951 and Y1175 are particularly important phosphorylation sites for endothelial cells. Y951 binds VEGFR-associated protein (VRAP), which is an important mediator of motility and survival [101]. Y1175 primarily binds PLC γ and SH2 domain-containing adaptor protein B (SHB), which are important mediators of proliferation [100, 102].

Similar to KIT, ligand binding induces receptor dimerization and autophosphorylation. Phosphorylation of the tyrosine residues creates docking sites for various signalling molecules, such as PLC γ and proteins containing SH2 (Src homology 2) domains [103]. PLC γ is responsible for activating protein kinase C (PKC), leading to the activation of the ERK1/2 cellular proliferation pathway [97, 100]. VEGFR-2 signalling is also responsible for initiating vascular permeability and proliferation, along with survival and migration of endothelial cells [104]. VEGFR-2 activity has also been shown to activate the PI3K/Akt and Bcl-2 anti-apoptotic pathways [105, 106]. The importance of this receptor was illustrated by studies showing VEGFR-2 $-/-$ mouse embryos were non-viable: death occurs due to improper development of

blood islands, endothelial cells and hematopoietic cells [107]. Due to their critical importance in normal physiological angiogenesis, VEGF and VEGFR-2 activity are often hijacked by neoplastic cells to advance tumour progression. Numerous human cancers have been shown to over-express VEGF, leading to paracrine signalling loops [108, 109]. VEGF produced by neoplastic cells binds to VEGFR2 on endothelial cells, and disrupts the delicate balance of pro and inhibitory angiogenic factors [109]. The end result is initiation of the angiogenic process to supply tumours with oxygen and nutrients. Autocrine VEGF/VEGFR signalling loops to promote cancer cell survival has also been observed repeatedly in breast cancer and melanoma studies [110-112].

RTK IN CANCER

It has been well established that dysregulated RTK activity contributes to the development of many human cancers. Constitutive activation of RTKs, such as KIT, can lead to constant activation of critical cell pathways, especially cell division and survival. Dysregulation can occur via different mechanisms, the most common being gene amplification, rearrangement, overexpression or mutation [28]. Activation of autocrine signalling loops can also initiate oncogenic RTK activity. This transformation mechanism has been described for both epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGF-IR) families [113-114]. Autocrine signalling loops basically involve a cell producing ligand, which is secreted to bind and activate cell surface growth receptors found on the originating cell. Intracrine and paracrine loops, where ligand production occurs within the cell and within the stroma, respectively, can also induce oncogenic RTK activity [28]. Colorectal cancer is an example of a human cancer that can be driven by signalling loops of VEGF binding to VEGFR2 [115]. Signalling loops and KIT dysregulation are often driving factors in the development of

gastrointestinal stromal tumours (GIST) [116]. Analysis of 36 GIST cases found 100% to be KIT-positive and 58% to co-express both the KIT receptor and the SCF ligand, with membrane bound SCF in concentrations 10 times higher than soluble levels [117]. This indicates continuous KIT activation and hence cell proliferation, either by activating KIT mutations or autocrine signalling loops of the KIT/SCF system [117].

KIT Dysregulation in Human Cancers

Irregular KIT expression and activity is associated with several human disorders. Loss or gain of function *c-kit* mutations are responsible for the development of autosomal-dominant piebaldism [118] and GIST [119]. Autocrine secretion of SCF leading to constitutive KIT activation has also been identified as driving factors for small cell lung carcinoma (SCLC), colorectal carcinoma, breast carcinoma, gynecological tumours and neuroblastomas [120]. Although rare (approximately 1000 to 8000 cases per year in the U.S), human mastocytosis is another KIT-induced cancer, characterized by uncontrolled mast cell proliferation [121]. KIT activation has also been implicated in the early stages of acute myeloid leukemia cell differentiation [122]. The combination of these disorders makes KIT an important therapeutic target, therefore studying KIT dysregulation in canine cancer models is an important task.

KIT Dysregulation in Canine MCT Cells

Abnormal KIT activation and signalling in canine MCTs is largely due to deletions or internal tandem duplications (ITDs) in the JM domain of exon 11 [12, 86, 123]. Other mutations are less commonly found in exons 8 and 9 [124] (See Figure 4). These gain-of-function mutations disrupt the auto-inhibitory function of the JM domain, allowing the KIT receptor to dimerize and stabilize even in the absence of SCF [125]. One study looking at 88 randomly

selected cases of canine MCTs found 9% to have ITD mutations in exon 11 [126]. This percentage can increase to 30-50% when looking at only intermediate or high grade MCT cases [127]. The majority of ITD mutations consist of in-frame duplications of 39-69 base pairs in length, and cause constitutive activation of KIT [85, 86, 128]. Webster *et al.* further showed that *c-kit* mutations were significantly associated with cytoplasmic KIT expression, high histological grade and decreased disease-free and overall survival times [123]. Further studies by Webster revealed that *c-kit* mutations and aberrant KIT localization was correlated to greater rates of neoplastic cells entering and progressing through the cell cycle [9]. This ultimately led to increased cellular proliferation and worse overall prognosis for MCT cases [9]. Recent research clearly implicates *c-kit* mutations and subsequent KIT activation as a significant factor in the development of canine MCT [129, 130]. Targeting of KIT should therefore be a priority for both veterinary oncologists and clinical researchers.

VEGFR2 Dysregulation in Human Cancers

Solid tumours cannot typically expand past a critical threshold size (1-2 mm³) due to inefficient diffusion of oxygen and nutrients. To circumvent this problem, many solid tumours begin secreting angiogenic factors such as VEGF. VEGF then binds primarily to VEGFR-2 on endothelial cells, which in turn stimulates survival, growth and proliferation [131, 132]. This leads to the formation of new vascular networks, thus supplying the solid tumours with the means to expand past the critical threshold size [133]. VEGF-induced angiogenesis is a critical step in the progression of many types of solid tumours, including colorectal cancer (CRC) [134], breast cancer [135] and SCLC [136]. Many cancerous cells additionally use VEGF autocrine/paracrine signalling loops as a survival mechanism [110-112]. For example, human melanoma cell lines harboured autocrine and intracrine VEGF/VEGFR-2 signalling loops as

evidenced by detection of native and phosphorylated VEGFR-2 in subcellular fractions [112]. VEGF and VEGFR-2 have consequently become intensely studied as anti-cancer targets. Many drugs have been developed to inhibit the ligand or receptor, with varying degrees of success. The common reports of VEGFR-2 signalling loops in human cancers and the highly conserved nature of this signalling pathway make it likely that canine cancers also utilize VEGFR-2 signalling to promote neoplastic development.

VEGFR2 Dysregulation in Canine MCT

Abnormal VEGFR2 signalling in tumour blood vessels has been implicated in many types of canine tumours, such as primary brain tumours [137] and cutaneous and squamous cell carcinomas [138]. While the role of VEGFR-2 in canine MCT is not fully understood [139], canine neoplastic mast cells are known to express both VEGF receptors and ligands [129, 130, 141]. Autocrine signalling loops have not yet been described, but it is likely that canine MCT development is partly dependent upon VEGF signalling activity. This hypothesis is supported by studies showing human and murine mast cells are known producers and secretors of VEGF [140]. This is important, since VEGF levels detected by immunohistochemistry in tumour samples are predictive of tumour progression and recurrence in dogs [142]. Rebuzzi *et al.*, (2007) found that immunostaining of tissue sections and slides revealed the vast majority of mast cells from grades II and III MCT tumours expressed VEGF, while only a smaller subset was found for grade I tumours [141]. All of these data make VEGF/VEGFR activity highly suspect in canine MCT development [142].

TARGETED CANCER THERAPY

History

Since Ulrich *et al.*, (1984) first linked a viral oncogene and mutated RTK activity to a human cancer, immense effort and resources have been allocated for the development of RTK inhibitors [143]. It is now widely known that aberrant RTK activity contributes to numerous human cancers. This effort has paid off, since effective RTK inhibitors are now a reality. RTK inhibition offers several advantages over traditional chemotherapeutics. First, they specifically target RTK signalling pathways typically used by neoplastic cells [28]. This results in far less toxicity and adverse side effects than chemotherapy drugs, which generally non-selectively target rapidly dividing cells [144]. RTK inhibitors may also sensitize neoplastic cells to chemotherapeutics, creating an effective double-punch system [145]. There are two general classes of RTK inhibitors, small molecule inhibitors and monoclonal antibodies and similar constructs (which can be further divided by the target of the neutralizing antibodies) [12]. The most successful strategy has involved the use of small molecule inhibitors, which block ATP binding sites via reversible or irreversible competitive binding [146]. This prevents RTK phosphorylation and activation of the associated signalling pathway. While ATP binding pockets are ubiquitous and required for physiological processes, side effects from these drugs are often minimal [12]. Much work has gone towards characterizing the binding pockets of target kinases, allowing researchers to build tailored inhibitors that have fewer off-target effects [12, 146]. RTK inhibitors are often easy to synthesize (once they have been developed), can easily traverse plasma membranes to reach intracellular targets and are orally bioavailable [12]. All of these advantages make RTK inhibitors an attractive treatment strategy.

Current Treatment Strategies for Human Cancers

Arguably the most successful TKI case story has been imatinib mesylate (Gleevec). This drug inhibits the constitutively active Bcr-Abl fusion kinase found in chronic myelogenous leukemia (CML) [147-149]. Imatinib has shown excellent clinical activity against CML, with remission induced in 95% of cases in the chronic phase [147-149]. This drug has also shown success against GIST, due to KIT inhibition. In several clinical trials, imatinib induced responses in 50-70% of patients, a response far greater than the ~5% rate induced by chemotherapeutics [12]. The highly conserved nature of ATP binding pockets means drugs such as imatinib can elicit effects against a multitude of RTK targets. This explains the positive effects of small molecule inhibitors being useful against different cancers, but can lead to off-target effects and toxicity [12]. Sunitinib is another successful small molecule inhibitor. This drug was developed to block a family of pro-angiogenic receptors, such as VEGFR, PDGFR and FGFR [150]. It also inhibits KIT to a lesser degree [12]. The anti-angiogenic activity of this drug makes it effective against neuroendocrine, colon and breast cancers and especially renal cell carcinoma [150].

The second class of RTK inhibitors, monoclonal antibodies, has also enjoyed clinical success. These drugs act by a variety of mechanisms. They can directly kill cells by binding to tumour cell surface receptors and initiating apoptotic pathways [151, 152]. Antibody binding can also prevent dimerization of cell surface receptors, thus neutralizing the cell by blocking the activation and signalling of critical cell pathways such as growth and proliferation [151, 152]. The drugs can also be used to bind to a cell and deliver a payload, such as a toxin, drug or radioisotope to kill or tag the cell for destruction [151]. Finally, monoclonal antibodies can be used for immune-mediated tumour cell destruction. This process involves antibodies attaching to

cells and acting as a red flag for phagocytes and T-cells, which promptly destroy the flagged cancer cell [151, 152].

Perhaps the best known example of a ligand disrupting monoclonal antibody is bevacizumab (Avastin). This drug neutralizes the VEGF ligand in humans, and has been a successful addition to combination therapy for treating CRC [153]. This drug continued the development trend of the earliest TKIs, namely to inhibit angiogenesis by disrupting the VEGF signalling pathway. The ideology behind this trend was that inhibiting angiogenesis would ultimately limit the growth of solid tumours, leaving them benign and of no medical concern. This thought process has changed somewhat recently, with much investigation of normalizing and enhancing blood vessel structure and flow to maximize delivery of chemotherapeutics to solid tumours [154]. Despite this shift, bevacizumab has been shown to be effective against non-small cell lung cancer (NSCLC) [155], metastatic renal cancer [156] and metastatic breast cancer [157].

Current Treatment Strategies for Canine Cancers

Much less data is available about the efficacy of RTK inhibitors in canines [12]. Most targets of monoclonal antibodies such as VEGF are only characterized for humans and not yet for dogs. Both small molecule inhibitors and monoclonal antibodies can also be very costly, lowering their potential utility [12]. However, there have been several successful applications of RTK inhibitors in veterinary oncology. The first canine trials of an inhibitor examined toceranib (Palladia) [158]. This drug has exhibited direct anti-tumour (decrease in tumour size and diameter) and anti-angiogenic activity (decrease in VEGFR-2 and PDGFR phosphorylation) due to VEGFR, PDGFR and KIT targeting [158]. The researchers consequently observed clinically

significant results in 54% of cases of a variety of cancers [158]. The highest response rate was observed in MCT cases (90% for cases with KIT mutations in exon 11 and 30% for cases without observable KIT mutations in exon 11) [158]. This was a breakthrough study, and had several important conclusions. First, it demonstrated that cases of canine MCT (especially cases with KIT mutations) stand to benefit significantly from RTK inhibition. Second, it demonstrated that RTK inhibition has potential application for a multitude of canine cancers such as sarcomas, carcinomas and melanomas. Finally, that RTK inhibitors can exhibit broad range effects against multiple RTK targets [158]. Follow up Palladia studies have continued the successful trend, with over 40% response rate for 145 cases of grade II or III non-resectable MCTs [159].

Masitinib is another RTK inhibitor that selectively and potently targets KIT [12, 80]. Studies have shown that masitinib inhibits the proliferation of a variety of mastocytoma cell lines [32]. Interestingly, masitinib was highly effective against MCT cell lines harbouring the V⁵⁵⁹D exon 11 mutation (IC₅₀ of 5 nM) but only weakly inhibited the growth of MCT cell lines with only the D⁸¹⁶V exon 17 mutation (IC₅₀ of 5 ± 2 μ M) [32]. The drug also inhibited tumour growth and increased overall survival time in mouse tumour models with subcutaneous MCT grafts [32]. In phase III trials conducted by Hahn *et al.*, (2008) of non-resectable or recurrent tumours, dogs treated with masitinib experience longer time to disease progression and increased overall survival than a placebo group [80]. There was also no significant difference in the incidence of adverse side effects between the groups (excluding vomiting and diarrhea), indicating masitinib is a safe and effective MCT treatment [80].

Disadvantages

Despite some cases of clinical success, RTK inhibitors exhibit several significant drawbacks. Quality of life is a critical factor in the decision making process for pet owners. Nearly all cancer drugs elicit side-effects, and RTK inhibitors are no exception. Due to their inherent function of targeting common receptors, they have some toxic effects on normal tissues. For example, toceranib can induce anorexia, vomiting, diarrhea, and gastro-intestinal bleeding [80, 158, 159]. Toxicity is often worse in cases of MCT, since higher circulating levels of histamine could lead to gastric or intestinal ulceration. Toceranib is also known to induce mild neutropenia and localized muscle cramping [12]. Due to the relatively new appearance of many inhibitor drugs, long term studies examining potential life-long side effects are also not yet available [12]. Toxicity profiles should therefore be considered at length, since it will have an effect on a pet's quality of life.

The lengthy development of RTK inhibitors is another disadvantage. It is extremely difficult and expensive to design and test RTK inhibitors. Making drugs that have specific targets is nearly impossible, since many RTK show sequence and structure similarity [160]. This means that 'selective' inhibitors will always have off-target effects, usually inhibiting a number of RTKs from the same family. For example, imatinib exhibits cardiotoxicity due to its inhibition of ABL [161]. The R&D cost associated with developing these drugs is also high. The average time from discovery to market is between 10-15 years, and the estimated average total costs of developing an oncology drug in the 1990's and early 2000's was \$1.042 billion [162]. The price of these drugs is consequently very high. Bevacizumab, despite low rates of success and highly variable clinical responses [163, 164], can cost as much as \$100,300 per year for breast cancer treatment [165].

A more concerning issue for RTK inhibitors is the transient nature of treatment response, due to acquired or inherent drug resistance [35, 163]. Neoplastic cells have a wide variety of potential resistance mechanisms at their disposal. Genetic mutations to the ATP binding pocket can effectively make an RTK inhibitor useless, since they are specifically tailored to bind to specific sequences [152]. For instance, human mastocytosis patients with the D⁸¹⁶V mutation are totally resistant to imatinib treatment [166-168]. Clonal expansion of a hardier subset of neoplastic cells can also repopulate a previously devastated tumour, creating a new and more aggressive neoplasm [152]. Other methods such as drug efflux pumps and relying on alternative signalling pathways could also contribute to resistance. Due to drug resistance and transient responses, RTK inhibitors are best used as part of a combination therapy with chemotherapeutics [152, 155]. While RTK inhibitors most certainly have therapeutic utility for treating a variety of human cancers, they leave much to be desired. Unintended toxicity, drug resistance and excessive cost are significant disadvantages for targeted therapy. Consequently, much effort has gone towards identifying pre-existing, FDA-approved drugs that exhibit novel anti-cancer effects. This effectively side-steps the R&D costs associated with oncology drugs, while still producing potentially viable cancer treatments that have significant clinical outcomes. One of the most promising discoveries of this effort has been itraconazole, which has repeatedly been shown to possess anti-angiogenic activity [39, 169].

ITRACONAZOLE

Anti-Fungal Properties

Itraconazole is FDA-approved in humans and widely prescribed off label for canines as an anti-fungal drug, belonging to the azole drug family [170]. It contains three nitrogens in the

azole ring, and is therefore termed a triazole. It has long been used as a safe and effective anti-fungal treatment with broad spectrum effects against superficial and systemic infections. Like other azole agents, itraconazole inhibits the fungal-mediated synthesis of ergosterol [170]. Specifically, itraconazole inhibits fungal lanosterol 14 α -demethylase (14DM), which catalyzes an essential step in the biosynthesis of ergosterol [170]. Ergosterol is a critical steroid component of fungal cell membranes, acting similarly to cholesterol in animal cell membranes [170]. Itraconazole therefore prevents ergosterol formation, ultimately disrupting membrane integrity and causing cell death [171]. The drug is well tolerated, with side effects mostly limited to nausea, vomiting, diarrhea and abdominal pain [170].

Anti-Angiogenic Effects

In addition to targeting ergosterol synthesis, itraconazole was recently and unexpectedly found to have potent anti-angiogenic activity [39, 169]. This discovery, along with a long history of safe application in humans and dogs, has motivated researchers to re-purpose itraconazole as a novel anti-cancer drug [39]. The drug has entered numerous human clinical trials, with success found against prostate cancer [172], basal cell carcinoma [173] and NSCLC [174]. Further trials of itraconazole treatment in breast cancer are also being conducted [175].

Most clinical trials have been based on data showing itraconazole potently inhibits endothelial cell proliferation. As previously discussed, endothelial cells are critical for angiogenesis. Itraconazole arrests the cell cycle of HUVEC and BAEC in the G1 phase [169], thereby preventing cell cycle progression and proliferation. This mechanism is likely responsible for the clinical success of itraconazole in treating numerous cancers that rely on angiogenesis to grow and metastasize. Previous research has indicated that 14DM may be important for

HUVEC/endothelial cell proliferation (as demonstrated in knockdown experiments by Chong *et al.*, 2007 [169]). Inhibiting 14DM could therefore be the mechanism disrupting angiogenesis. However, it is still unclear if 14DM is the major target in endothelial cells. There are conflicting reports about itraconazole's wide potency range against human 14DM synthesis (with LC50 values ranging from 0.61 to 30 μ M) [176, 177], indicating 14DM may not represent itraconazole's viable therapeutic target. More recent research has demonstrated that other azole agents that target 14DM synthesis have no effect on endothelial cell proliferation [39]. This information strongly indicates that itraconazole's anti-angiogenic activity is possibly unique, may not involve 14DM, and occurs through a mechanism yet to be identified.

Possible mechanisms recently proposed to explain this anti-angiogenic activity include inducing an accumulation of cholesterol in late endosomes/lysosomes, thereby disrupting protein trafficking [178]. Inhibition of mTOR, a kinase that regulates a host of cellular functions, has also been proposed [178]. The most prominent theory however, comes from studies showing disruption of the late stages of N-glycosylation in macrophages treated with itraconazole [179, 180].

Receptor N-glycosylation

N-linked glycosylation is a form of post-translational modification (PTM) whereby glycan oligosaccharides are attached to the nitrogen atoms of asparagine residues [181, 182]. Glycosylation is an important PTM, and is absolutely critical for proper protein folding and function. It is required for protein stability, and protection from proteases that would otherwise degrade the protein [183, 184]. Glycosylation begins on the rER, where an elaborate and complex process biosynthesizes an oligosaccharide of three glucose, nine mannose and two N-

acetylglucosamine residues. The oligosaccharide is then transferred to a nascent protein in the ER lumen [184]. The protein-linked oligosaccharide then undergoes trimming by glycosidases and mannosidases in the Golgi to remove the glucose and some mannose residues [184]. Glycosyltransferases can add sugar residues to the oligosaccharide, allowing eukaryotic cells to produce a wide array of different glycosylation patterns on proteins such as RTKs [184]. The trimming and processing additionally ensures proper protein folding and subsequent passing of quality control checks [184].

The three general types of glycan patterns produced by this process are high mannose, complex, and hybrid glycans [182, 184]. High mannose glycans are simply two N-acetylglucosamines with numerous mannose residues added on. Complex glycans can contain any combination of saccharide, usually forming large and diverse oligosaccharides. Hybrid glycans contain a high mannose branch and a complex branch. In addition to folding and function, glycan residues are critical for intracellular protein trafficking and transport [182, 184]. Receptor glycosylation has also been shown to be critical for ligand binding, since sugar residues can act as recognition isotopes [185]. Researchers have described N-linked glycosylation as a primary factor influencing ligand binding, with ligand-receptor interactions significantly decreasing after altering glycan patterns via sialylation [185]. Disruption of glycosylation patterns can therefore have severe consequences for protein activity. This is an important fact, since targeting the glycosylation process could theoretically disrupt proteins important for cancer progression.

Glycosylation and Cancer

The glycosylation process is critical for cellular proteins, including those proteins linked to cancer. Therapeutic targeting of glycosylation could therefore be used as a supplement to traditional chemotherapeutics and RTK inhibition. This could theoretically create a triple effect treatment, whereby rapidly dividing cells, ligand or RTK binding pockets, and protein glycosylation (and therefore trafficking and function) are targeted. Recent research by Nacev *et al.* (2011) has found that itraconazole treatment significantly disrupts glycosylation patterns in HUVEC cells [39]. Nacev serendipitously discovered that VEGFR2 from cells treated with itraconazole exhibited irregular migration patterns on SDS-PAGE gels. Specifically, they found that itraconazole induced the accumulation of a low molecular weight species of VEGFR2 [39].

VEGFR2 typically appears on PAGE gels as a double-banded protein. The top band of 230 kDa represents mature, complex glycosylated protein while the lower band of 200 kDa represents immature, less glycosylated protein [39]. Nacev found that after itraconazole treatment, the top band was erased and the lower band was enhanced. Global glycan experiments discovered a significant loss of complex glycans, while IP and IF experiments showed hypoglycosylated VEGFR2 localizing to the Golgi [39]. This indicates that receptor trafficking has been severely disrupted by itraconazole, leading to protein retention in the Golgi and decreased receptor expression at the cell surface. This was likely the cause for supplemental findings that hypoglycosylation of VEGFR2 significantly affected signalling. VEGFR2 phosphorylation was decreased by 80% and phosphorylation of PLC γ 1 (a downstream signalling target of VEGFR2) was decreased by 60% [39]. The signalling downregulation led to a decrease in endothelial cell proliferation and survival. This effect was also unique to itraconazole, since no other member of the same azole family had any effect on receptor glycosylation [39]. They

additionally found that itraconazole induced hypoglycosylation of EGFR in a renal cell carcinoma cell line [39]. This finding is significant, since it shows that itraconazole's effects are not limited to endothelial cells or non-neoplastic cells.

Itraconazole's exact mechanism of glycosylation disruption is not yet known. There are three primary theories, each concerned with different spatial and temporal stages of protein glycosylation and trafficking. The first involves the calnexin/calreticulin chaperone system. Calnexin (a transmembrane protein) and calreticulin (a soluble protein) are lectins in the ER responsible for premature protein degradation due to improper folding. It is possible that itraconazole alters protein glycan patterns in the ER, causing the proteins to be tagged for degradation [39]. The second involves retention in the Golgi during the processing and trimming steps. It is possible that itraconazole completely alters the protein glycans at this step, causing the protein to become trapped in the Golgi. This could explain why VEGFR2 was localizing to the Golgi after itraconazole treatment, since the packaging and transportation steps cannot be initiated [39]. The final theory involves disruption of vesicle glycosylation, which is required for proper intracellular trafficking. This would consequently lead to a backlog of proteins in the Golgi waiting to be packaged, and a decrease in cell surface expression. Both of these results were seen in experiments conducted by Nacev *et al.* [39].

Disrupting receptor glycosylation could therefore hold great promise for anti-cancer therapy. Cancers driven by RTK dysregulation could be especially vulnerable, particularly when treated with a combination of itraconazole, TKI, and chemotherapy. The effect of itraconazole against endothelial cells and widely conserved RTK such as VEGFR2 warrants further investigation for many types of cancer. Inhibition of N-glycosylation in macrophages [179, 180] also indicates itraconazole could have effects in other immune system cells, such as canine MCT

cells. This was the rationale for this project, where cell signalling inhibition by itraconazole was investigated in two canine MCT cell lines.

CANINE MCT CELL LINES MCT1 AND MCT2

A major problem with studying canine MCT *in vitro* is the lack of suitable cell lines. Neoplastic mast cells are notoriously hard to isolate from primary tissues, with purification and maintenance further complicating the process [186, 187]. Consequently, the majority of canine MCT studies have been conducted on two cell lines, C2 and BR [188, 189]. These cell lines were generated decades ago, and were passaged through mice to enhance tumorigenicity. For these reasons, both the C2 and BR cell lines may not accurately represent the true behaviour of MCT response to treatment. To partially remedy this situation, effort has gone towards generating new cell lines, namely the CL1 [187] and MPT-1 [190] cell lines. A major component of this project also involves extracting neoplastic cells from primary tumour samples, with the ultimate aim to generate novel, immortal cell lines (MCT3-MCTn). Until such time as a new cell line was potentially generated, this project used two novel cell lines generated by Thompson *et al.*, (2012) in an attempt to diversify the canine MCT research, MCT1 and MCT2 [191].

Both cell lines were derived from MCT samples provided by the Ontario Veterinary College, University of Guelph, small animal surgical oncology service. MCT1 was generated from a grade III MCT from a 7 year old male castrated Shar-pei, with evidence of metastasis to the mandibular lymph node [191]. The MCT was characterized as non-encapsulated, poorly demarcated and infiltrating deep into subcutaneous tissue. Histology reports indicate the tumour was densely cellular and exhibited a high degree of pleomorphism and high mitotic rate [191]. Doubling time was approximately 48 hours for MCT1 without additional exogenous ligand.

Importantly, MCT1 has no reported mutation in the *c-kit* sequence. As previously discussed, MCT with ITD mutations in exon 11 typically have a greater response to targeted therapy than non-mutants [158]. This could explain why MCT1 is more resistant to toceranib treatment, with pKIT inhibition only significant at extremely high doses (10 μ M). It is important to note that the tumour from which MCT1 was derived did harbour the 48 bp ITD mutation in exon 11. The loss of the ITD mutation from tumour to cell line could have been a result of allele loss, allele mutation, or tumour heterogeneity. The contrast from the originating sample shows that cell lines cannot completely represent an *in vivo* neoplasm, since tumours will invariably have heterogeneous populations and variable biological behaviour.

MCT2 was generated from a grade I MCT from a 7 year old female spayed Boxer with no evidence of metastasis [191]. The MCT was characterized as densely cellular with well differentiated, highly granular mast cells. Doubling time was approximately 72 hours without additional ligand. In contrast to MCT1, MCT2 exhibits a 48 bp ITD within exon 11 of *c-kit*. This mutation confers constitutive KIT activation via disruption of the JM domain. MCT2 is significantly more susceptible to toceranib inhibition, with effects on pKIT observed at doses as low as 0.01-0.1 μ M. Toceranib affected VEGFR2 phosphorylation in both cell lines. However, neither cell line had observable changes in native KIT or VEGFR2 expression or glycosylation compared to untreated control cells.

Both cell lines show autophosphorylation of KIT in the absence of exogenous ligand, showing phosphorylation of both immature (non-glycosylated) and mature (glycosylated) bands. Both cell lines show autophosphorylation of VEGFR2 in the absence of exogenous ligand. MCT1 and MCT2 additionally produce high levels of VEGF. The autophosphorylation and VEGF production strongly indicates that autocrine signalling loops play a significant role in

MCT1/2 survival [191, 192]. Other groups have generated similar findings when observing autocrine signalling loops in canine mammary carcinomas, making the previous conclusion plausible [193, 194]. However, this has yet to be definitively established for canine MCT cells. Hence, further studies are required to completely identify the major pathways of MCT development.

Table 1. Histological grading of canine mast cell tumours developed by Patnaik *et al.*, 1984.

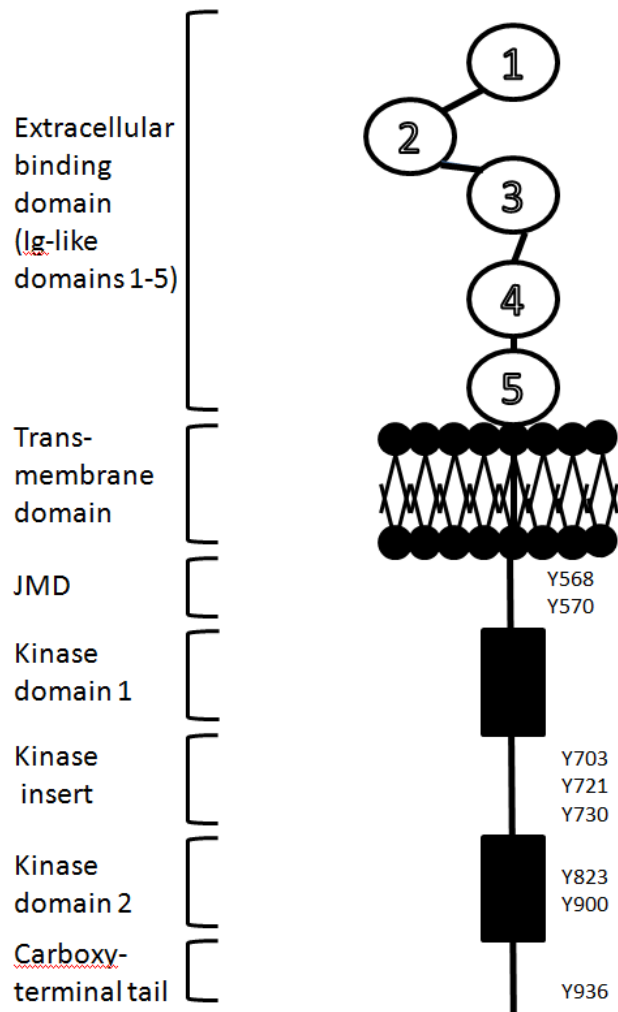
Grade	I	II	III
Tumour location and cellular characteristics	Dermis. Mast cells sparse and separated by ECM	Dermis and subcutis. Moderate to highly cellular	Invades deep into subcutaneous tissue. Highly cellular
Cellular morphology	Round, monomorphic, distinct cell borders, medium sized granules	Round to ovoid, moderate pleomorphism, fine granules, distinct borders	Medium sized, round, ovoid, or spindle shaped pleomorphic cells, indistinct borders, fine granules or none present
Nuclear morphology	Round	Round to indented, single nuclei for the majority	Indented, vesicular nuclei with one or more nucleoli, scattered multinucleate cells

Source: adapted from [22].

Table 2. World Health Organization staging and classification for mast cell tumours.

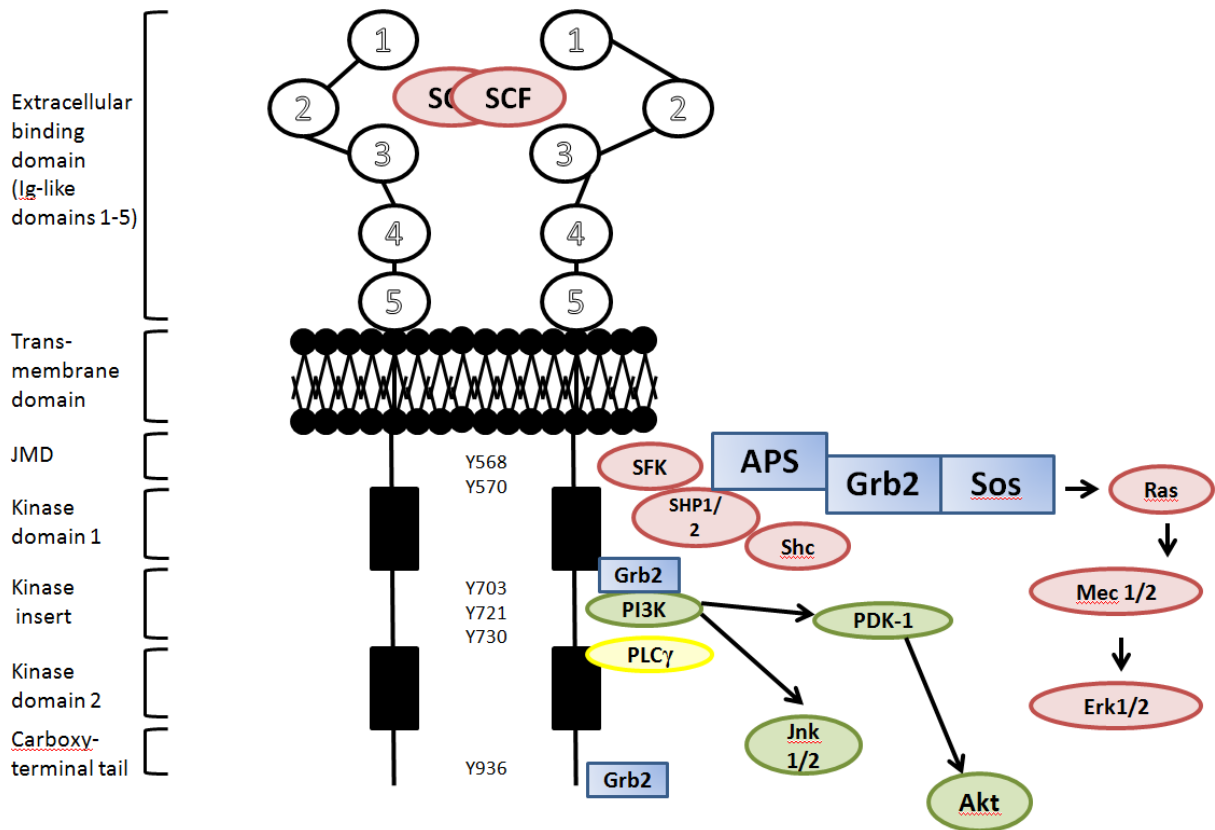
Stage	Description
0	One tumour incompletely excised from the dermis, without regional lymph node involvement a: Without systemic signs b: With systemic signs
I	One tumour confined to the dermis, without regional lymph node involvement a: Without systemic signs b: With systemic signs
II	One tumour incompletely excised from the dermis, with regional lymph node involvement a: Without systemic signs b: With systemic signs
III	Multiple dermal tumours, or large infiltrating tumour, with regional lymph node involvement a: Without systemic signs b: With systemic signs
IV	Any tumour with distant metastases or recurrence with metastases

Source: adapted from [20].



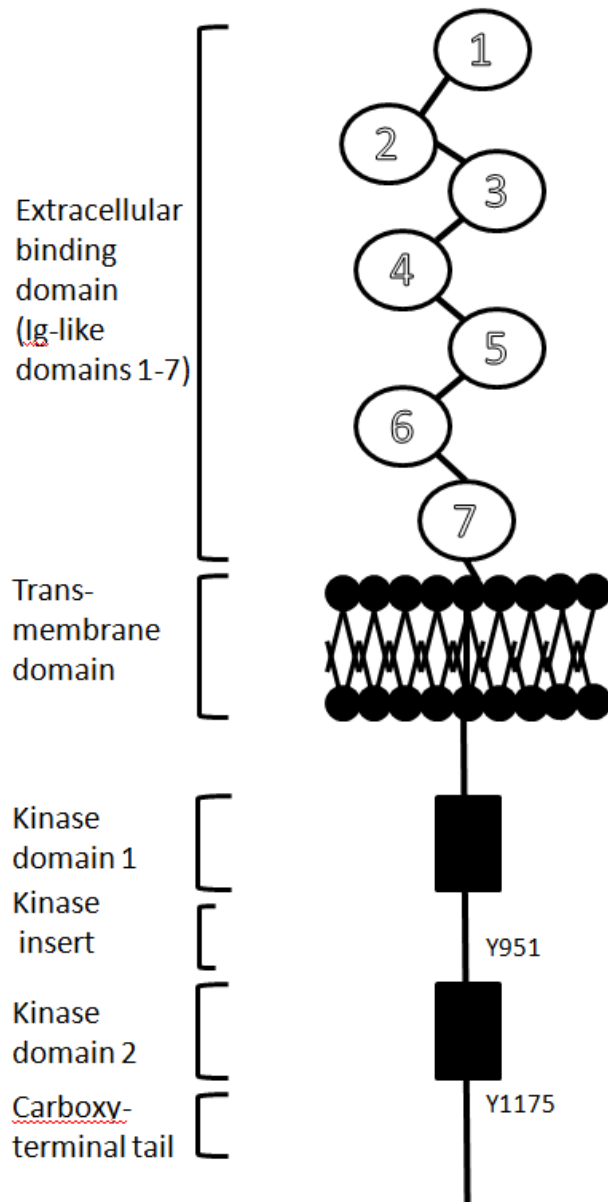
Source: adapted from [84].

Figure 1. Schematic representation of a monomeric KIT receptor. Tyrosine residues important for cell signalling pathways indicated. Abbreviation: Ig, immunoglobulin.



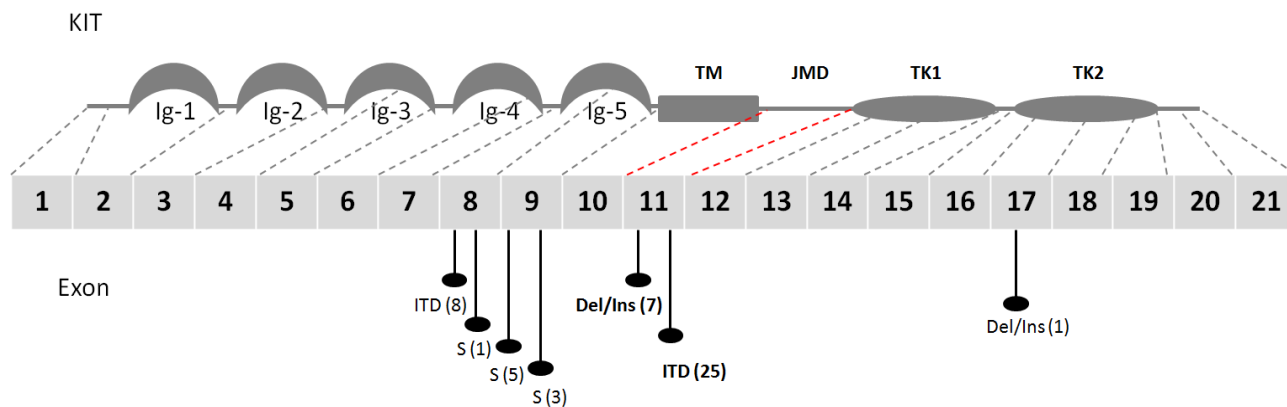
Source: adapted from [84].

Figure 2. Schematic representation of a dimeric KIT activation by stem cell factor (SCF). Also shown and summarized are the signalling proteins recruited to phosphorylation sites by KIT activation. Sites Y568 and Y570 recruit Src family kinases, sites Y703 and Y936 recruit Grb2, site Y721 recruits PI3K and site Y730 recruits PLCγ. Major activated pathways include Ras/Erk (enhances cell proliferation, growth, differentiation, and survival), PI3K/Akt (reduces apoptosis) and SFK (initiates cell cycle progression, survival and protein trafficking). Abbreviation: Ig, immunoglobulin.



Source: adapted from [14]

Figure 3. Schematic representation of a monomeric VEGFR2 receptor. Tyrosine residues important for cell signalling pathways in endothelial cells are indicated. Abbreviation: Ig, immunoglobulin.



Source: adapted from [85].

Figure 4. Schematic representation showing location and frequency of mutations within *c-kit* exons for 191 cases of canine MCT. Dashed lines indicate the portion of the KIT receptor encoded by the indicated exon. 26.2% of all cases had observable mutations, with 64% of mutations found in Exon 11 (highlighted by dashed red lines). Abbreviations: S, substitution, ITD, internal tandem duplication, Del/Ins, deletion/insertion, Ig, immunoglobulin, TM, transmembrane domain, JMD, juxtamembrane domain, TK1/2, tyrosine kinase 1/2.

RATIONALE

Cell signalling inhibition holds great promise for current and future cancer treatment. Investigating the cellular level of RTK activity is critical for understanding the link between dysregulated RTK and cancer progression. Carrying out studies that determine drug inhibition profiles and uncovering potential resistance mechanisms is therefore critical for enhancing future human treatment. An excellent model for these studies is canine MCTs, as they are spontaneous, available in high numbers, exhibit dysregulated RTK and correlated targeted therapy resistance and closely resemble human pathways of cancer development.

Objective 1: Investigate receptor inhibition in canine MCT cells using a variety of TKIs and the novel anti-cancer drug, itraconazole.

Building on previous work showing aberrant RTK localization and activity can cause canine MCT, the activity of several TKIs will be tested against MCT1 and MCT2. The primary outcomes will be assessed using changes in proliferation rates and phosphorylation status. The localization of both KIT and VEGFR2 in response to treatment will also be assessed using IF.

Objective 2: Assess the change, if any, in glycosylation status of VEGFR2 and KIT in response to itraconazole treatment.

Glycosylation is critical for proper protein folding and function. Targeted disruption of this process could have a significant impact on neoplastic cells and their ability to survive and proliferate. The effect of itraconazole on KIT and VEGFR2 glycosylation will be qualitatively analyzed via Western Blotting, IP and glycosylation assays.

Objective 3: Attempt to generate novel MCT cell lines from primary tissues, in order to expand our currently limited panel of immortalized, continuous canine cell lines.

As discussed previously, a serious impediment to studying RTK inhibition in canine MCT cells is a lack of available cell lines. This issue will be addressed by obtaining primary tumour samples from the OVC clinic and attempting to isolate, purify and sustain novel cell lines. This will provide more cell lines to investigate mutational statuses, TKI strategies, and potential resistance mechanisms. Furthermore, a wider variety of continuous canine cell lines will better represent the heterogeneous nature of a neoplastic tumour, thus increasing the impact and relevance of *in vitro* studies.

MATERIALS AND METHODS

A list of suppliers for chemicals and reagents can be found in Appendix I and details of solution preparations can be found in Appendix II.

Tissue culture

Canine MCT cell lines MCT1 and MCT2 were grown in standard cell culture conditions. Cells were maintained in a 37°C humidified incubator with 5% CO₂ in single, 10 cm plates. Cells were maintained in standard RPMI 1640 culture media supplemented with 10% FBS and 1% sodium pyruvate. Culture media was changed every 1-2 days and passaged every 3-4 days. Cell collection for MCT1 involved removing used media, and washing with 4 mL of sterile PBS. Cells were then treated with 3 mL of trypsin for ~5 minutes or until detachment occurred. Collected cells and trypsin were then added to 3 mL of fresh RPMI 1640 with 10% FBS to deactivate the trypsin. Cells were pelleted at 350 \times g for 4 minutes. Cell collection for non-adherent MCT2 involved collecting into 15 mL tubes, pelleting at 350 \times g for 4 minutes, resuspension in 5 mL PBS and pelleting at 350 \times g for 4 minutes.

Protein extraction and quantification

Cell pellets produced by the previous described method were stored at -80°C or used immediately for protein extraction. Whole cell lysate (WCL) for adherent MCT1 cells was collected by the following protocol. Cells were left in monolayer and treated with a lysis buffer (containing 1mM PMSF, and 2 µg/mL of aprotinin and protease inhibitor cocktail 2) for 1-3 minutes. Cells were collected by cell scrapers, with lysis buffer containing WCL and cell debris were pipetted into microcentrifuge tubes. Cell lysates were incubated on ice for 10 minutes prior

to centrifugation at $15,000 \times g$ for 15 minutes at 4°C . Supernatant was collected and separated into aliquots.

Whole cell lysate (WCL) for non-adherent MCT2 cells was collected by the following protocol. Cells pellets (as collected by the previously discussed protocol) were resuspended in 40-80 μL of lysis buffer, depending on pellet size. Cell lysate was then incubated on ice for 10 minutes, transferred to a microcentrifuge tube and centrifuged at $15,000 \times g$ for 15 minutes at 4°C . Supernatant was collected and separated into aliquots. When analyzing protein for phosphorylation in Western blots, cells were pretreated with 0.5 mM sodium orthovanadate for 8 minutes prior to cell lysis (this preserves the phosphorylation status). When analyzing KIT phosphorylation, cells were pretreated with 1 $\mu\text{L}/\text{mL}$ of rcSCF for 20 minutes prior to cell lysis. Protein was quantified using the Bio-Rad DCTM Protein Assay Kit, OD630. Values of each sample were standardized to control values for 0.2, 0.5, 1, 2.5, and 5 $\mu\text{g}/\mu\text{L}$ BSA.

SDS-PAGE and western immunoblotting

Protein samples were prepared with MilliQ water and 8X Sample buffer to a final volume of 24 μL . Samples were then placed in a 95°C heating block for 5 minutes. The protein was then loaded into 7.5% polyacrylamide gels and separated by electrophoresis in electrophoresis running buffer at 125 V for ~1 hour and 45 minutes. Gels containing separated samples were then equilibrated in transfer buffer for 20 minutes. Separated protein was then transferred onto a methanol-activated polyvinylidene difluoride (PVDF) membrane for 2 hours at 100 V. Successful transfers were confirmed by staining with amido black, which was removed from the membrane by washing with methanol followed by MilliQ water. Depending on protein of interest, membranes were then blocked for 1 hour at room temperature with either 5% non-fat

milk in Tris-buffered saline/Tween 20 (TBS-T) or 5% bovine serum albumin (BSA) in TBS-T. The membranes would be incubated in 5% milk when protein of interest was non-phosphorylated and 5% BSA when protein of interest was phosphorylated. Membranes were then incubated on a shaker overnight at 4°C with primary antibodies diluted in 5% milk or 5% BSA in TBS-T.

Primary antibodies (raised against human targets) and their concentrations were as follows: mouse-anti- α -tubulin (1:400,000), rabbit-anti-phospho-cKIT for Y719 (1: 2,000), rabbit-anti-cKIT (1: 2,000), rabbit-anti-phospho-VEGFR2 for Y951 (1: 2,000), rabbit-anti-VEGFR2 (1: 2,000), mouse-anti-caspase 3 (1: 1000). After overnight primary incubation, membranes were washed three times for 5-10 minutes in TBS-T and incubated in HRP-labeled goat-anti-mouse or goat-anti-rabbit secondary antibodies (1:20,000) in either 5% milk in TBS-T (for native or non-phosphorylated protein) or 4% milk/1% BSA in TBS-T (for phosphorylated protein) for 1 hour at room temperature. After secondary incubation, membranes were washed three times for 10-15 minutes in TBS-T. Membrane bands were visualized in a Bio-Rad ChemiDocTM XRS+ system using chemiluminescent HRP substrate LuminataTM Forte. Band molecular weights were determined via comparison to GeneDirex[®] BLUeye Prestained Protein Ladder. Band denistometry was analyzed using the Bio-Rad Image LabTM Software.

Semi-dry transfer was used when detecting protein of low molecular weight (<50 kDa). The same protocol for wet transfer was used, with the following alterations. Semi-dry transfer buffer in a semi-dry transfer apparatus was used; proteins were transferred at 16 V for 30 minutes.

Crystal violet cell number assay

Adherent MCT1 cells (100 μ L with 5000 cells/well) were plated in 96 well plates and treated for various time periods. After treatment period, media was discarded and cells were incubated in equal volumes of formalin for 20 minutes on a shaker (to fix cells in place and prevent lifting). Formalin was discarded and cells were incubated in equal volumes of crystal violet stain for 10-20 minutes on a shaker. Stain was discarded; wells were washed with MilliQ water and allowed to air dry overnight at room temperature. Cells were finally incubated with 100 μ L of acetic acid for 10 minutes on a shaker to dissolve crystals. Plates were read in a spectrophotometer at 630 nm and the absorbance values were recorded and compared to the absorbance value of 5000 cells to determine change in cell population.

MTT cell growth assays

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assays were carried out for both adherent MCT1 and non-adherent MCT2. Briefly, this protocol follows the same set up as crystal violet assays, with 100 μ L containing 5000 cells/well. After treatment, the cells are incubated in 10 μ L of MTT solution for 3-4 hours. This solution will form purple crystals in response to mitochondrial activity. The crystals are then dissolved and read in a spectrophotometer, with high values indicating high mitochondrial activity and a robust cell population and low values indicating absent or decreased mitochondrial activity. Hence mitochondrial activity is used as a proxy estimate for cell growth and general activity. Published protocols from Sigma Aldrich Cell Growth Determination Kit MTT Based[®] were used with the following caveat:

Phenol-red free RPMI1640 media was used for MCT2 cell culture, as phenol dye could have altered spectrophotometer results.

Co-Immunoprecipitation

For co-immunoprecipitation, magnetic beads coupled to antibodies were used to isolate and purify protein of interest. The first step in the protocol was the coupling of antibody to the magnetic beads. This was accomplished using a kit and published protocols from Life Technologies Dynabeads® Antibody Coupling Kit with the following alteration: 5 µL of antibody was used per mg of magnetic beads. For the co-immunoprecipitation step, WCL from treated cells were incubated with the antibody coupled to magnetic beads. This extracted the protein of interest from the WCL. The protein was then eluted from the antibody and bead mixture, leaving pure and isolated protein for subsequent glycoprotein analysis. Published protocols from Life Technologies Dynabeads® Antibody Coupling Kit (detergent lysis method) was used, with the following alterations: Extraction Buffer contained 20 µL of PMSF and 6 mg NaCl, bead and cell lysate solution was incubated for 40 minutes, resuspension of Elution Buffer and beads was incubated for 10 minutes on a high RPM pulsator.

Glycosylation assay

A kit and published protocols from Invitrogen Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit were used to assess receptor glycosylation. The following alterations were made to the protocol: both 150 and 75 mm SDS-PAGE gels were used, gel solution was filtered prior to use, number of wash steps were maximized with times increased by 25%, the gel was fixed overnight in the second fixing solution step, 28 µL of protein sample/well was loaded for 75 mm gels and 48 µL of protein sample/well was loaded for 1.5 cm gels and gels were stained for a

minimum of 2 hours. Gels were then stained with SYPRO Ruby total protein stain using standard protocols.

Immunofluorescence

Adherent cells were grown either on sterile Fisherbrand Superfrost® Plus slides, or regular cell culture plates (which would be collected via trypsinization, counted and ~50,000 cells cytopun onto slides). After treatment, cells were fixed with 4% paraformaldehyde for 15 minutes followed by washes with PBS (3 x 5 minutes). Cells were then permeabilized in PBS Triton X- 100 for 5 minutes. Slides were then incubated in 5% Normal Goat serum and Dako Serum Block solution for 1 hour. Following blocking, cells were incubated in darkness overnight at 4°C in 1:100 anti-cKIT antibody, anti-phospho-cKIT antibody and/or fluorescently tagged Alexa Fluor 488 Conjugate anti-cKIT mAb (all subsequent incubation periods were done in darkness). Slides were then washed in PBS (3 x 5 minutes) and incubated at room temperature for 1 hour with 1:200 goat-anti-rabbit secondary antibody conjugated to Cy3®. Cells were then washed in PBS (3 x 5 minutes) and incubated at room temperature in 30 µM DAPI nuclear stain for 5 minutes (30 µM DAPI was diluted to 1:100). Finally, cells were washed in PBS (3 x 5 minutes), 1-2 drops of DAKO fluorescent mounting media were added per area of interest followed by glass coverslips.

Non-adherent cells were grown in regular tissue culture plates, collected via centrifugation, resuspended and cytopun onto slides. The same protocol detailed above was used from this step on. Images were captured using a Leica Microsystems DMLB100T microscope with 40X objective. Brightness and contrast of images was then enhanced using Adobe Photoshop®.

Novel cell line generation

Immediately before beginning, 2.0 mg/mL of collagenase solution was prepared in 10 mL of serum-free RPMI 1640. Samples were immersed briefly in ethanol to disinfect outer surface. The sample was then minced into small pieces (1-2 mm²) by scissors and scalpel. Tumour pieces were then placed into 50 mL conical tube containing the collagenase solution and placed in a 200 rpm oscillator for 2 hours at 37°C. The digested pieces were then passaged through 16g, 18g, and 20g needles to further break up cell clumps. The cell suspension was then passed through a 40 µm mesh to remove large cells and debris. Finally, the suspension was pelleted at 350 g for 4 minutes, resuspended in RPMI 1640 with 10% FBS and antibiotics, plated in poly-L lysine high adhesion plates and placed in a 37°C incubator.

Statistical analysis

Statistical analysis was performed using GraphPad Prism® software. Non-parametric Kruskal-Wallis tests were used to determine if differences existed between treatments. Mann-Whitney tests were used to determine if differences existed between two treatment groups. At least three biological replicates were used for each statistical analysis, and treatments were considered significantly different if a p-value less than 0.05 was generated for the data.

RESULTS

KIT and VEGFR2 expression in response to itraconazole

Two cell lines, MCT1 and MCT2, were treated with itraconazole (0, 100, 200, 400, 800 nM) for 24 hours. Cellular response was analyzed by western blots and densitometry of phosphorylated and native KIT and VEGFR2. Itraconazole did not appear to induce any changes in MCT1 receptors. After 24 hours, there was no significant downregulation of pKIT and no change in native KIT expression in MCT1 cells (Figure 5A and C). Similar to KIT, both activated and native forms of VEGFR2 showed no change compared to the control after 24 hours of treatment (Figure 6A and C).

However, MCT2 showed remarkable susceptibility to itraconazole (Figure 5B and D). Even at low doses (200 nM), there was a shift in the migration patterns of KIT. The upper band, representing mature glycosylated protein, begins to shift towards the lower band, representing immature protein. The shifting process is largely completed for higher doses (400 and 800 nM). This shift was mirrored by KIT phosphorylation, which was abolished for the upper band and qualitatively downregulated overall. Similar to KIT, VEGFR2 in MCT2 cells showed susceptibility to itraconazole treatment (Figure 6B and D). At low doses (200 nM), native VEGFR2 begins to shift towards the lower band, with the upper band largely abolished at doses of 400 nM or greater. Total phosphorylation was also qualitatively downregulated at the highest dose (800 nM).

KIT and VEGFR2 expression in response to toceranib

In order to achieve more satisfactory downregulation of phosphorylation, both cell lines were treated with itraconazole and toceranib (solely and in combination) during an 8 hour time course (Figure 7). For MCT1 cells, KIT phosphorylation was not affected by itraconazole but

was significantly abolished as early as 2 hours after treatment with 1.0 μ M toceranib.

Toceranib's inhibitory effect of phosphorylation continued for the entire time course of this experiment. Native KIT expression stayed largely consistent compared to control, with a slight upregulation in cells treatment with single agent toceranib (Figure 7A).

Similar to MCT1, KIT phosphorylation in MCT2 cells was not affected by itraconazole and phosphorylation was abolished as early as 2 hours after toceranib treatment (Figure 7B). Toceranib's inhibitory effect of phosphorylation continued for the entire time course experiment, with negligible recovery seen at 8 hours. Interestingly, treatment with toceranib also led to a marked increase in native KIT expression.

Toceranib's inhibitory effect on phosphorylation was not limited to KIT, since pVEGFR2 was also disrupted (Figure 8). Recall from Figure 6A that low doses of itraconazole had no effect on pVEGFR2. Figure 8B shows that toceranib clearly downregulates VEGFR2 phosphorylation (in the presence of low dose itraconazole) in MCT2 cells.

Cellular growth

The effect of drug treatment and subsequent disruption of post-translational modifications in both cell lines was evaluated by estimating cell number. Figures 9 and 10 show the characterization of three compounds, itraconazole, toceranib and masitinib and their effects on the proliferation of MCT1 cells over 72 hours of treatment. For itraconazole, only high dose treatment (800 nM) showed significant decreases in total cell number at 24, 48 and 72 hours. For the remainder of the treatment doses, itraconazole largely inhibited proliferation past the original 5000 cell population but did not cause cell numbers to decrease beyond the starting plating density (Figure 9A).

For toceranib, inhibition of proliferation was seen at doses as low as 100 nM (Figure 9B). The optimal dose appeared to be 1.0 μ M, since it rapidly inhibited proliferation (24 hrs), and caused a drop in cell number by 72 hours, although this was not significant. Doses of 10 μ M, while apparently deadly to MCT1 cells, are pharmacologically irrelevant [195] and only included to determine what dose was required to kill a majority of the cells.

MCT1 cells were largely resistant to masitinib treatment (Figure 10A). Even high doses of 400 nM after 72 hours of treatment did not significantly inhibit proliferation or cause reductions in cell number.

Combination therapies were also examined to determine if any additive, antagonistic or synergistic relationships existed between drugs. Combination itraconazole and masitinib inhibited cell proliferation and caused a minor reduction in cell number after 72 hours (Figure 10B), but this was not significant. Combination itraconazole and toceranib was more effective, with proliferation halted but non-significant reductions in cell number observed after 48 hours. Combination masitinib and toceranib was less effective, although proliferation was still inhibited (likely due to toceranib more so than masitinib, since the decreases in cell number are similar to single agent toceranib seen in Figure 9B). Finally, triple combination treatment was the most effective at reducing cell numbers to approximately 30% of the starting population (Figure 10B). The results seen with triple combination were significant compared to itraconazole and masitinib treatment. The results for MCT1 were repeated with crystal violet assays (Figure 11), which show similar patterns. Masitinib was largely ineffective, single agent itraconazole and single agent toceranib inhibited proliferation and lowered cell numbers (only at highest doses and after longest treatment period, but these differences were not statistically significant), while combination itraconazole and toceranib had the greatest effect.

The same drug characterization was performed against MCT2, using the same optimal doses for toceranib and itraconazole (Figure 12). Unlike MCT1, MCT2 cells appeared far more susceptible to masitinib treatment. After 72 hours of treatment, 400 nM treatment reduced cell numbers by ~50%. (This indicates a halt in proliferation and possibly cell death). MCT2 cells were also more susceptible to itraconazole treatment, with cell numbers reduced by ~50% after 72 hours of treatment. Toceranib had similar effects, with cell numbers reduced by ~65% after 72 hours. Similar to MCT1, the most effective dose appeared to be combination itraconazole and toceranib (reduced cell number by approximately ~70%). This dose significantly reduced cell numbers and the addition of masitinib achieved negligible additional cell number reduction.

Caspase-3 activation

To determine if toceranib and itraconazole were inducing reductions in cell number via apoptosis, western blots were performed to examine activation of caspase-3 (cleaved caspase-3, indicative of apoptosis). Figure 13 shows that MCT1 caspase cleavage was present but negligible for toceranib and combination treatment. MCT2 cells exhibited significant caspase cleavage for toceranib treatments, with slight caspase activation for high dose itraconazole. To better capture levels and timing of caspase activation, 48 hour time course experiments were performed. Figure 14 shows western blots of MCT1 cells treated with four treatment groups after four time points. For all the treatment groups caspase activation was clearly negligible, if detectable at all. It should also be noted that cleaved caspase-3 was not seen in the etoposide positive control for MCT1 cells. MCT2 cells continued to show caspase activation in response to toceranib treatment, with levels clearly upregulated sometime between 12 and 24 hours and lasting throughout the experiment (Figure 15).

KIT glycosylation

To further examine the effect of itraconazole on RTK glycosylation, immunoprecipitation and glyco-analysis of KIT from MCT1 and MCT2 was performed. Figure 16 shows WCL from both cell lines after low and high dose itraconazole treatment. This image is an SDS-PAGE gel stained with Pro-Q Emerald glycoprotein dye, with stained bands representing glycosylated protein. After 24 hours of treatment, it is clear there is no apparent change in global protein glycosylation in either cell line. Figure 17 shows immunoprecipitated KIT receptor, stained with glycoprotein and total protein dye. For MCT1, the IP process was likely not efficient enough, since no glycosylated bands can be seen in the zero drug control (Figure 17A and 17B). However, MCT2 shows a key elimination of the upper band of glycosylated protein in the itraconazole treated receptor (Figure 17A and 17B, highlighted by red box). Total protein stains also show a decrease in protein on treated KIT receptor compared to untreated KIT. This further indicates that itraconazole is having an effect on the normal KIT production processes in MCT2 cells.

Immunofluorescence

IF images were taken of each cell line after 24 hours of treatment, in order to identify qualitative changes in KIT activation and localization in response to treatment. For MCT1 cells, itraconazole treatment appeared to induce a more stippled to focal perinuclear localization of KIT. This effect was not seen to the same extent with toceranib treatment, but was replicated during the combination treatment, along with a change in nuclear morphology (with many cell nuclei shrinking and taking on abnormal shape) (Figure 18).

For MCT2 cells, native KIT expression also exhibited noticeable changes. After itraconazole treatment, much of the cytosolic KIT localization is qualitatively abolished or partly replaced by perimembranous localization. This effect on KIT localization is also seen with toceranib and combination treatment (Figure 19). Importantly, toceranib and combination treatment lead to the formation of numerous apoptotic bodies in MCT2 cells.

Novel cell line generation

One of the objectives of this project was to attempt the generation of additional novel canine MCT cell lines, a notoriously difficult task [186, 187]. Most attempts at isolating and sustaining neoplastic cells from primary tumour samples failed or generated very short term cultures. Over the course of 2 years, 16 attempts were made to isolate neoplastic mast cells from primary tumours. Approximately 25% yielded mast cell populations that persisted into short term cultures, but never in populations large or pure enough for characterization. However, one tumour sample yielded the most densely cellular and persistent populations of neoplastic mast cells (Figure 18). These co-cultures of fibroblasts and neoplastic mast cells persisted for weeks, with careful removal of waste and addition of media and growth supplements. Although the cells eventually died off, numerous healthy populations were cryopreserved. These populations can be characterized or grown at any time in the future, or until such a time that new or more effective protocols are developed to sustain canine MCT cells. This could possibly allow these cells to be sustained into an immortal cell line under the right circumstances.

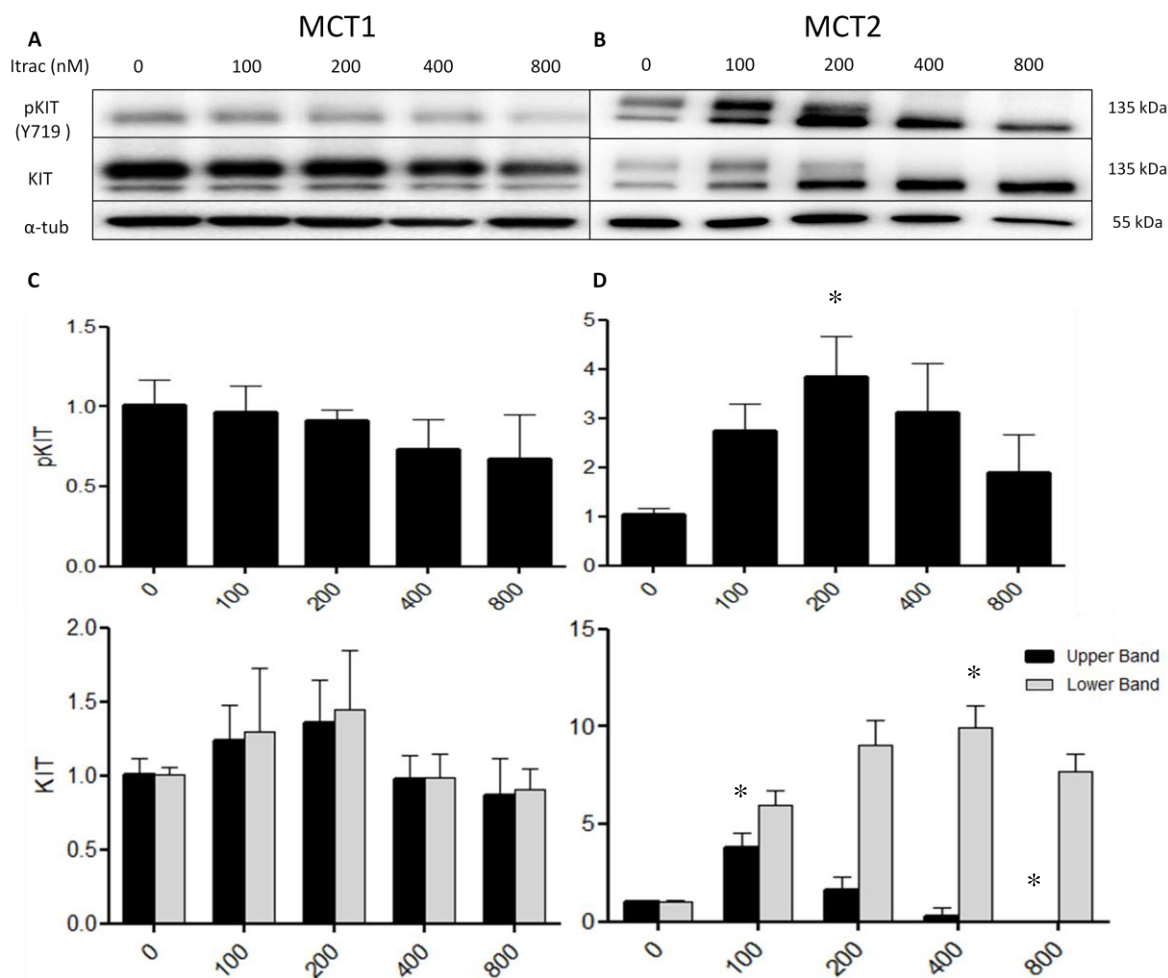


Figure 5. Western blots and densitometry of KIT in MCT cells treated with itraconazole for 24 h. Blot showing activated and native KIT response to itraconazole in MCT1 cells (A) and MCT2 cells (B). KIT in MCT2 cells show a shift towards the lower band, indicating a higher proportion of immature protein glycosylation. (C) shows densitometry values for pKIT and KIT in MCT1 cells, while (D) shows densitometry values for pKIT and KIT in MCT2 cells (total pKIT is normalized to α -tubulin and each KIT band is relative to total pKIT, normalized to α -tubulin). Significant differences compared to respective control are noted by a * ($p < 0.05$). Abbrev. Itrac=itraconazole, α -tub= α -tubulin. N= 3

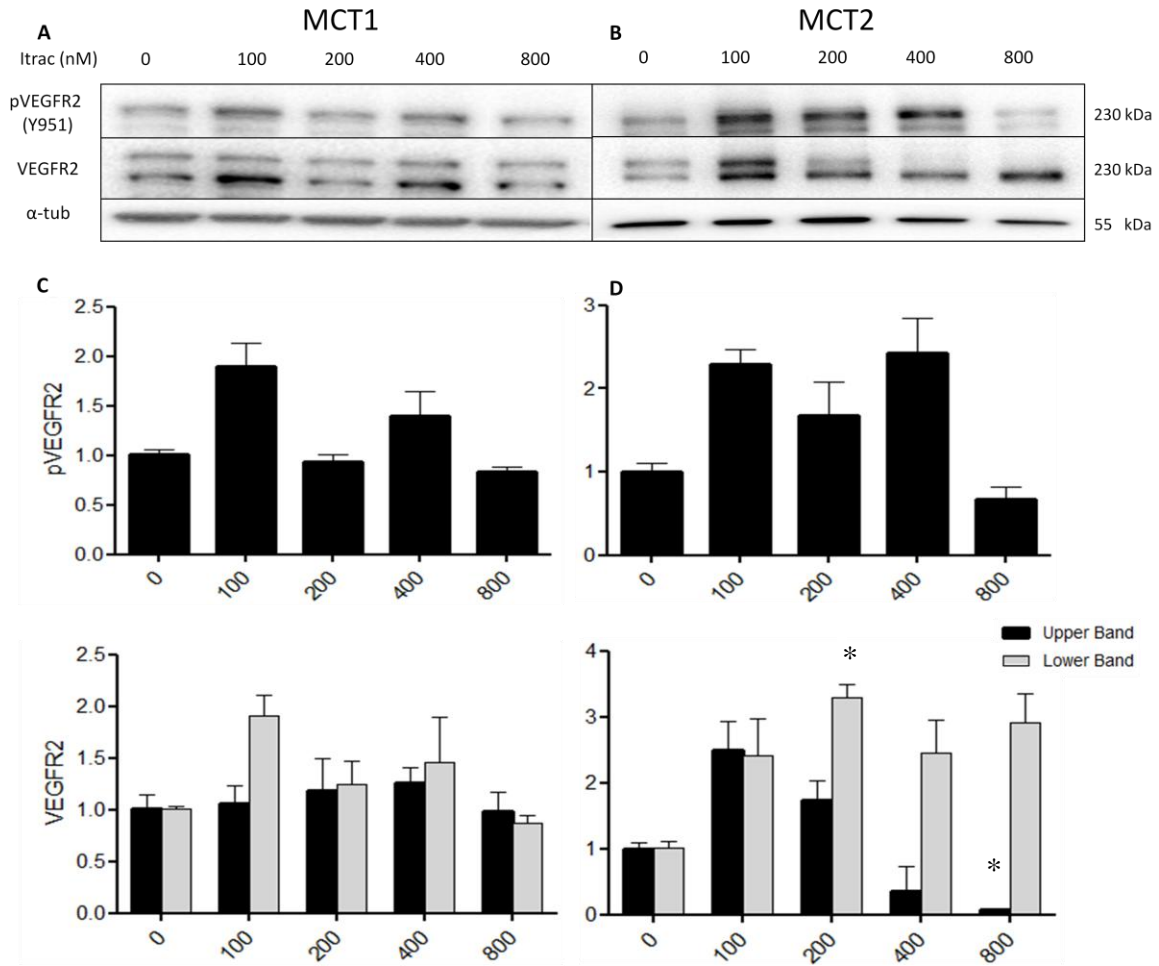


Figure 6. Western blots and densitometry of VEGFR2 in MCT cells treated with itraconazole for 24 h. Blot showing activated and native VEGFR2 response to itraconazole in MCT1 cells (A) and MCT2 cells (B). VEGFR2 in MCT2 cells show a shift towards the lower band, indicating a higher proportion of immature protein glycosylation. (C) shows densitometry values for pVEGFR2 and VEGFR2 in MCT1 cells, while (D) shows densitometry values for pVEGFR2 and VEGFR2 in MCT2 cells (total pVEGFR2 is normalized to α -tubulin and each VEGFR2 band is relative to total pVEGFR2, normalized to α -tubulin). Significant differences compared to respective control are noted by a * ($p < 0.05$). Abbrev. Itrac=itraconazole, α -tub= α -tubulin. N= 3

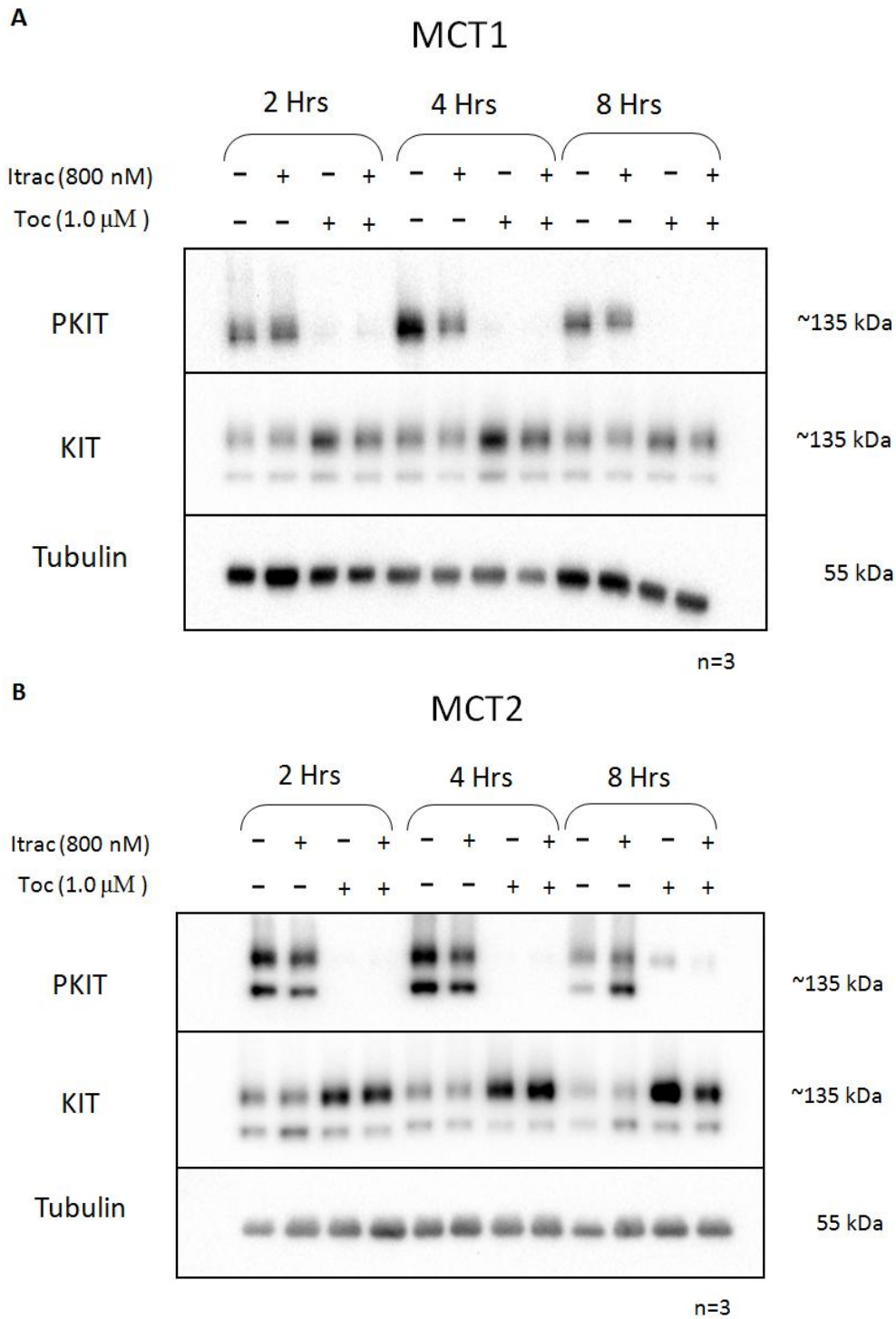


Figure 7. Western blots showing disruption of KIT phosphorylation over 8 h time-course in MCT cells treated with toceranib and itraconazole. (A) shows KIT response in MCT1 cells to four different treatment groups (no drug control, itraconazole, toceranib, and combination itraconazole/toceranib) over 8 h of treatment. (B) shows KIT response in MCT2 cells to the same treatment groups over 8 h of treatment. Abbrev. Toc= toceranib. N= 2

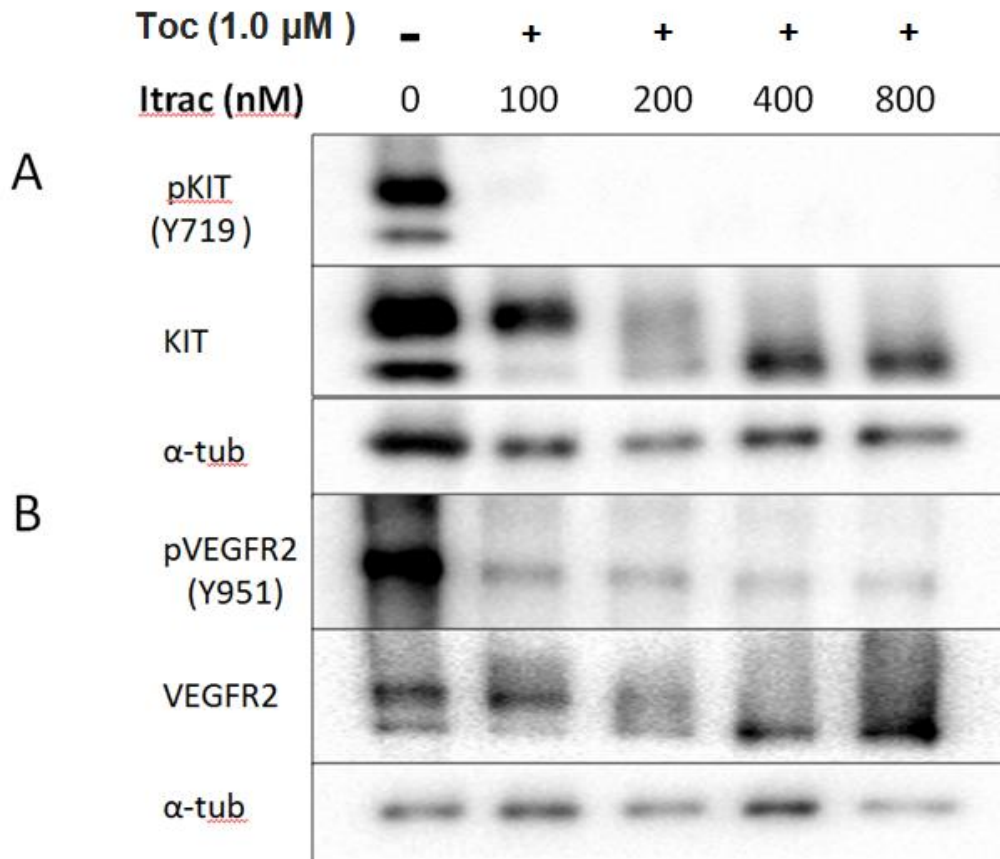


Figure 8. Western blot of KIT and VEGFR2 in MCT2 cells treated with combination itraconazole and toceranib for 48 h. Doses of itraconazole vary from 0-800 nM, while toceranib dose remains constant at 1.0 μ M. (A) shows pKIT and KIT response to combination treatment, with phosphorylation eradicated and upper band glycosylated protein abolished. (B) shows pVEGFR2 and VEGFR2 response to combination treatment, with phosphorylation drastically reduced and upper band glycosylated protein abolished. N=1

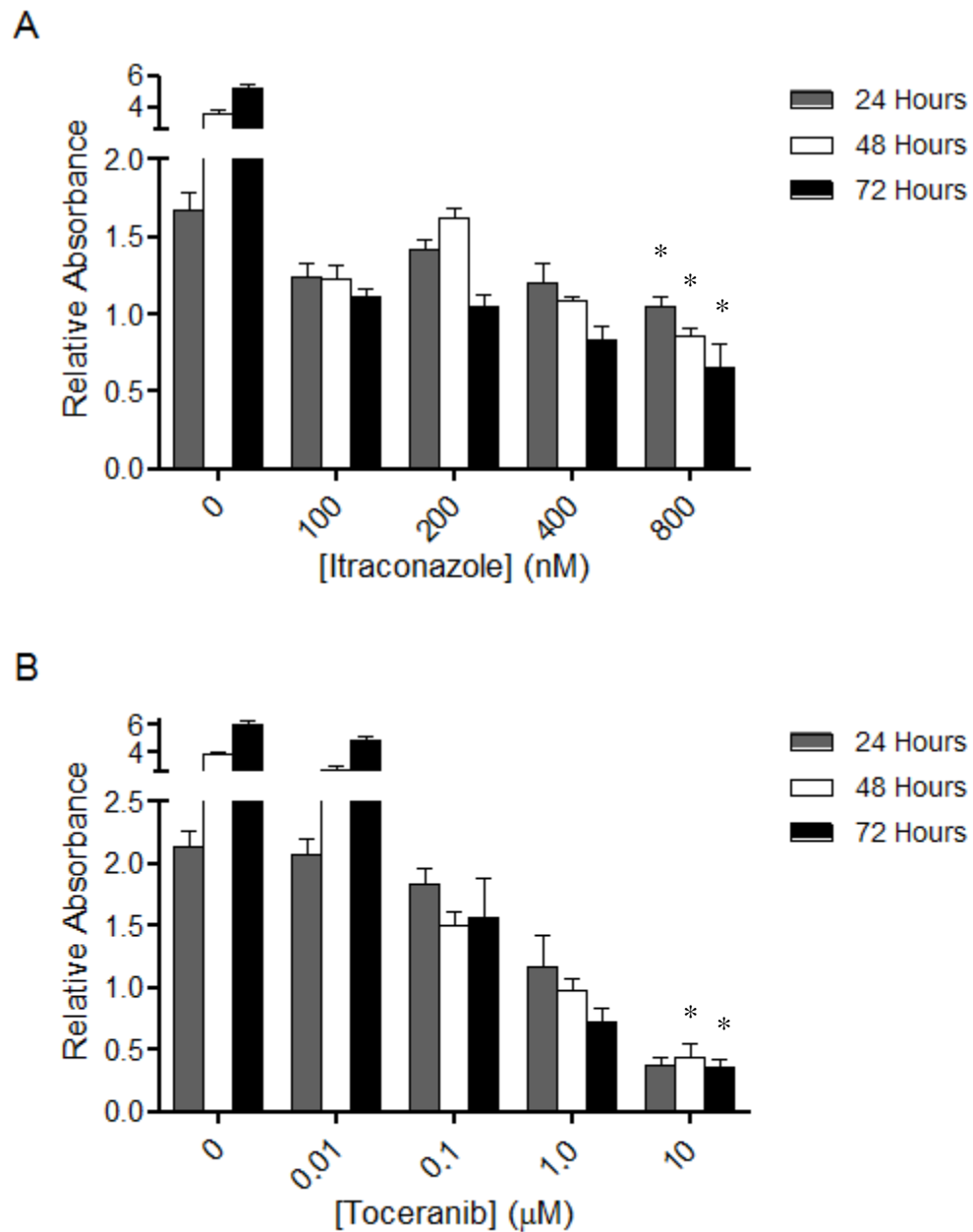


Figure 9. Cell number assay of MCT1 cells treated with itraconazole or toceranib for 24, 48 or 72 h. MTT assays were used for adherent MCT1 cells, to keep data consistent with MTT assays used for MCT2. All data have been normalized to starting absorbance of 5000 cells. (A) shows dose response of itraconazole over 72 hours, (B) shows dose response of toceranib over 72 hours, with significant differences compared to control indicated by a * ($p < 0.05$). (N=3)

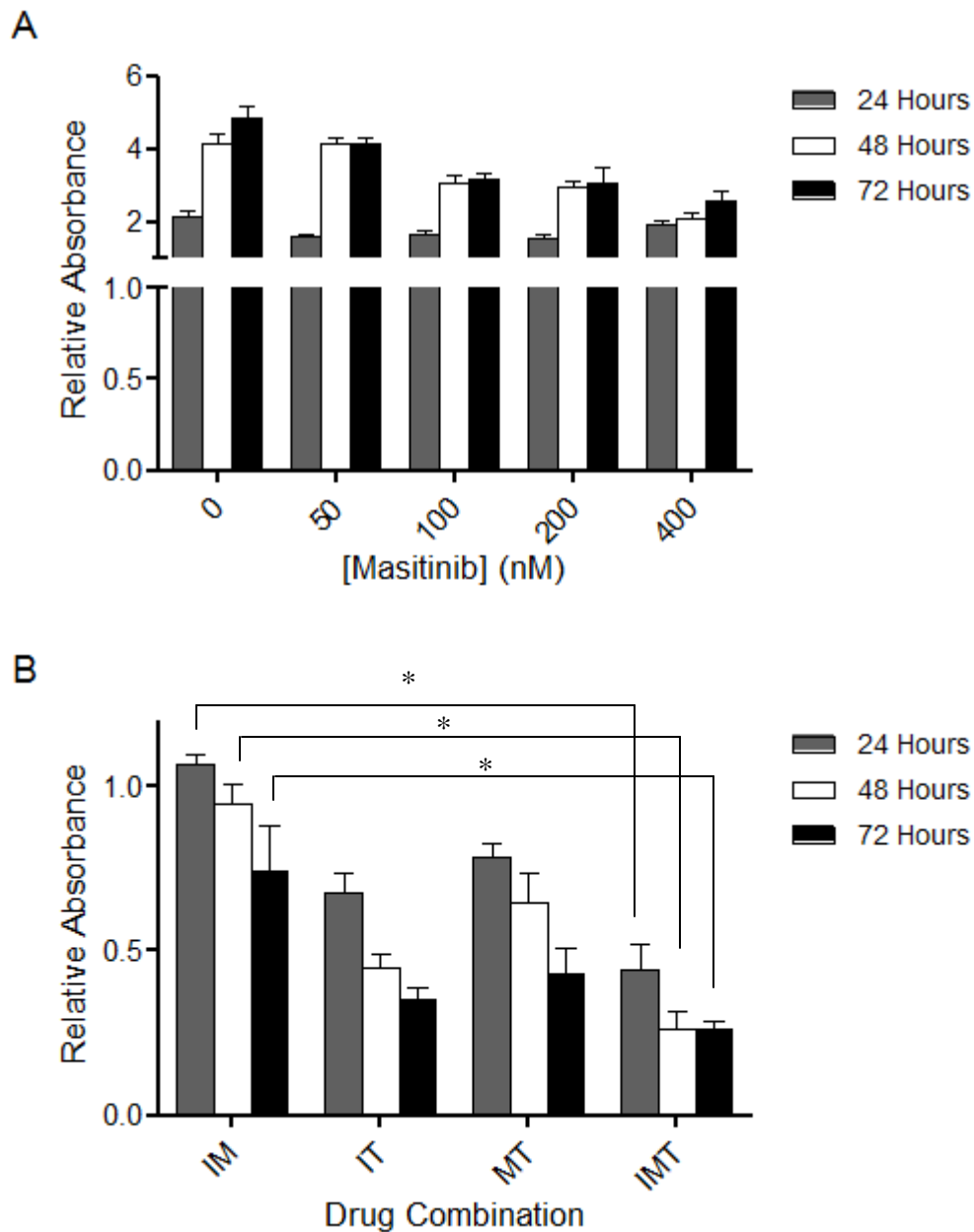


Figure 10. Cell number assay of MCT1 cells treated with various drugs for 24, 48 or 72 h. MTT assays were used for adherent MCT1 cells, to keep data consistent with MTT assays used for MCT2. All data have been normalized to starting absorbance of 5000 cells. (A) shows dose response of masitinib over 72 hours and (B) shows combination therapy with itraconazole (800nM), toceranib (1.0 μ M) and masitinib (400 nM). Significant differences between treatment groups are indicated by a * ($p < 0.05$). Abbrev. I= itraconazole, T= toceranib, M= masitinib. (N=3)

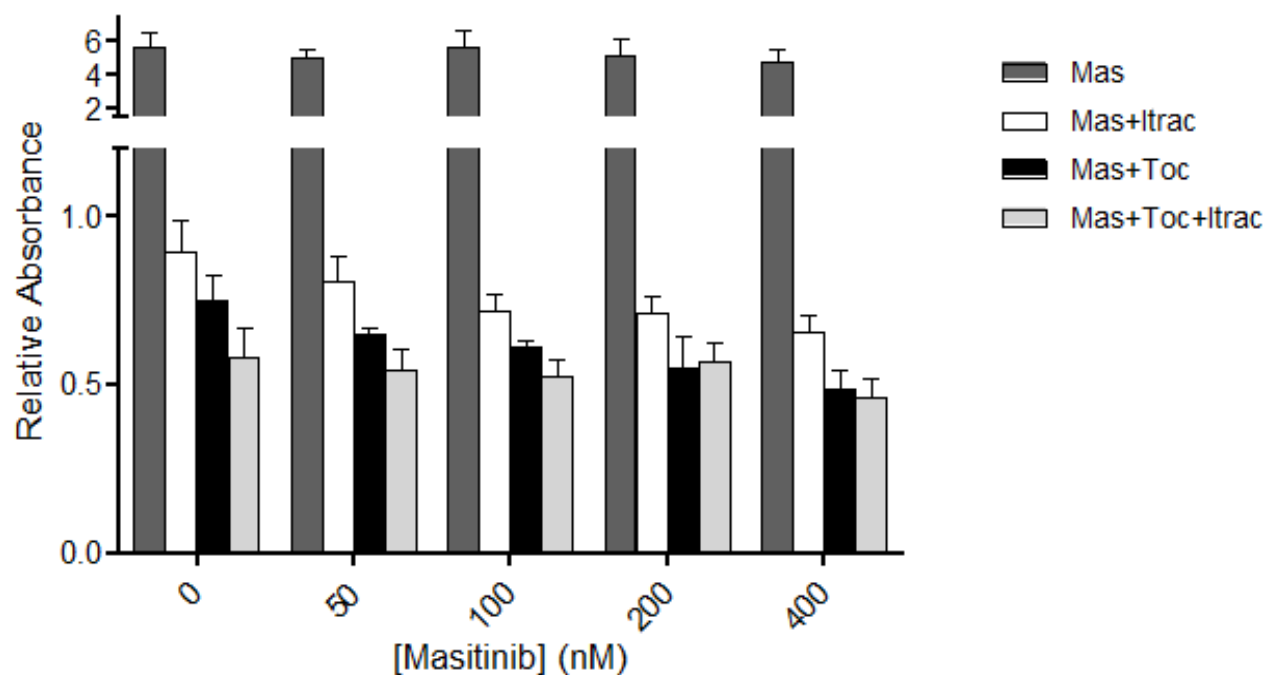


Figure 11. Cell number assay of MCT1 cells treated with various drugs for 72 h. Crystal violet assays were used for adherent MCT1 cells, with all data normalized to starting absorbance of 5000 cells. Doses of itraconazole (800nM) and toceranib (1.0 μ M) remained constant, while masitinib dose changes from 0-400 nM. For all treatments, addition of other drugs produced significant differences in cell number reduction compared to single agent masitinib treatment. Abbrev. Mas= masitinib. (N=3)

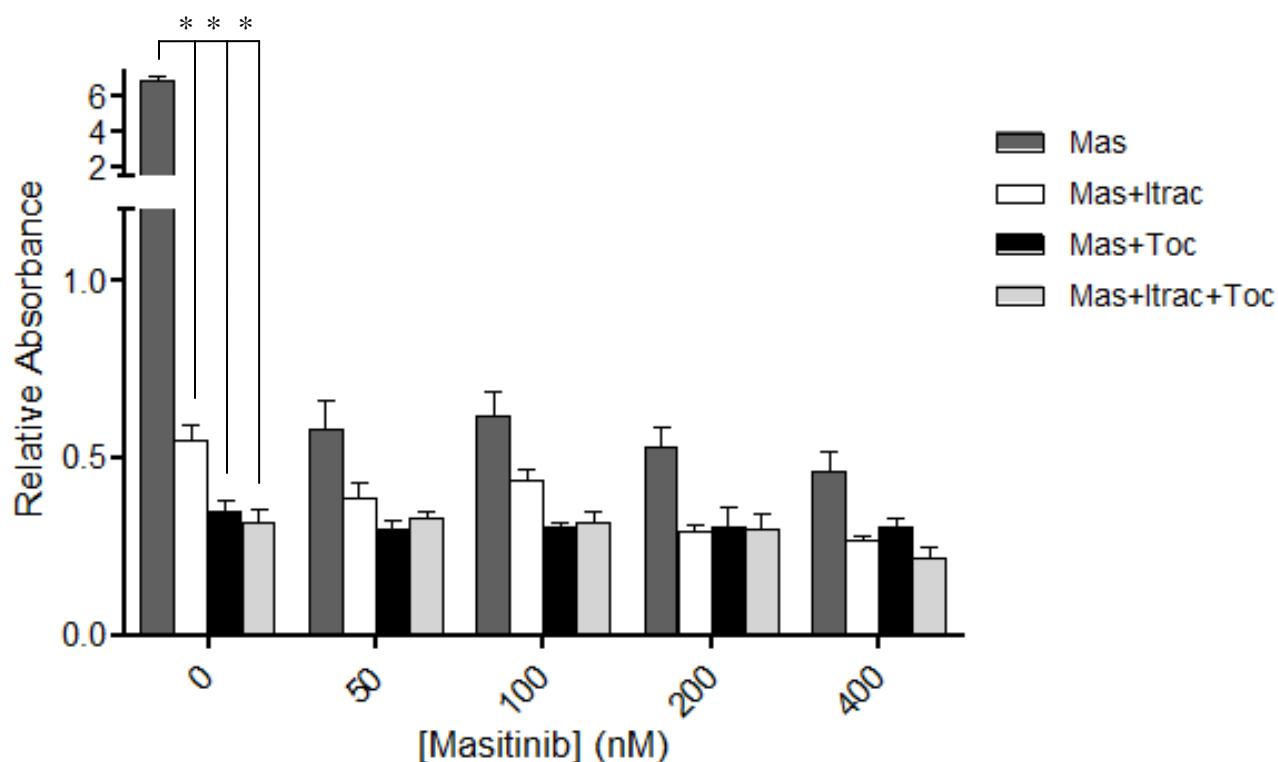


Figure 12. Cell number assay of MCT2 cells treated with various drugs for 72 h. MTT assays were used for non-adherent MCT2 cells, with all data normalized to starting absorbance of 5000 cells. Doses of itraconazole (800nM) and toceranib (1.0 μ M) remained constant, while masitinib dose changes from 0-400 nM. Significant differences between treatment groups are indicated by a * ($p < 0.05$). (N=3)

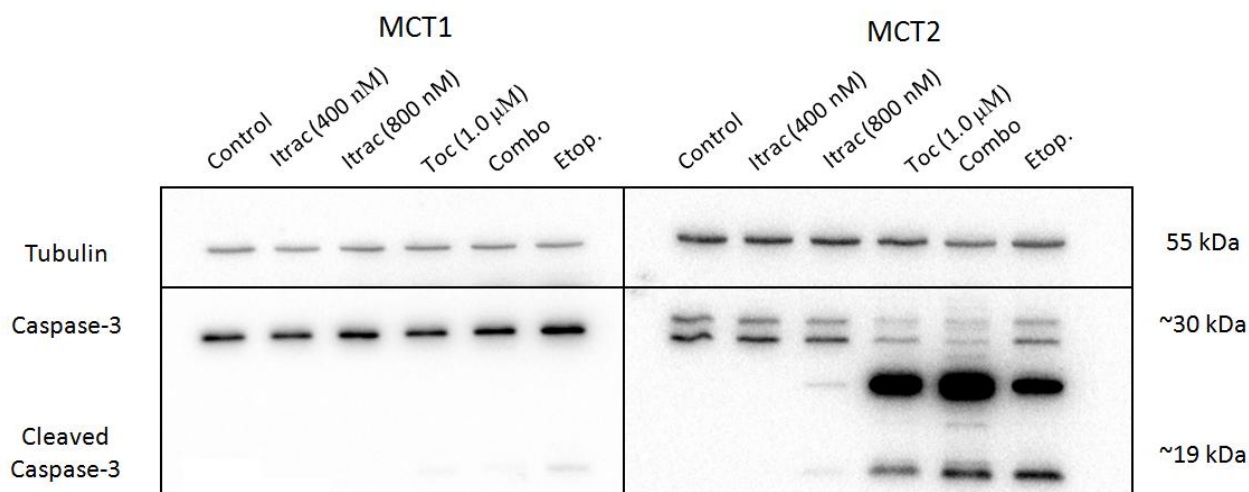


Figure 13. Western blots showing caspase activation in MCT cells in response to various drug treatments for 24 h. Six treatment groups (negative no-drug control, low dose itraconazole, high dose itraconazole, toceranib, combination high dose itraconazole/toceranib and positive control etoposide) were used to determine caspase activation. N=2

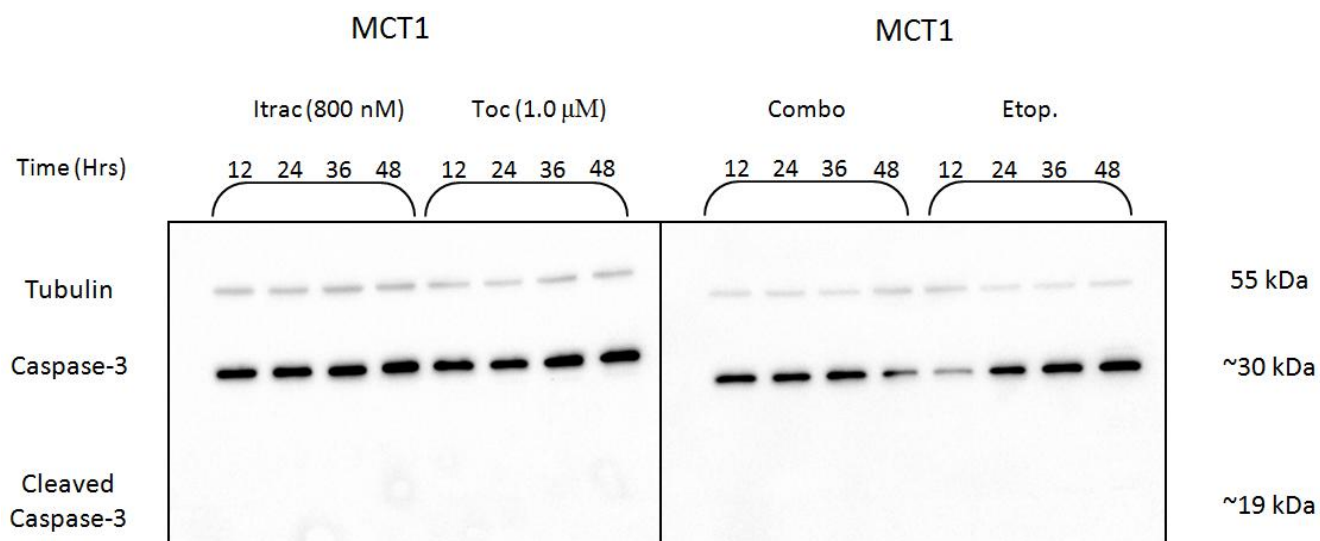


Figure 14. Western blots showing caspase activation in MCT1 cells in response to time course drug treatment. Four treatment groups (itraconazole, toceranib, combination itraconazole/toceranib and positive control etoposide) and four time points (12, 24, 36 and 48 hours) were used to determine caspase activation. N=3

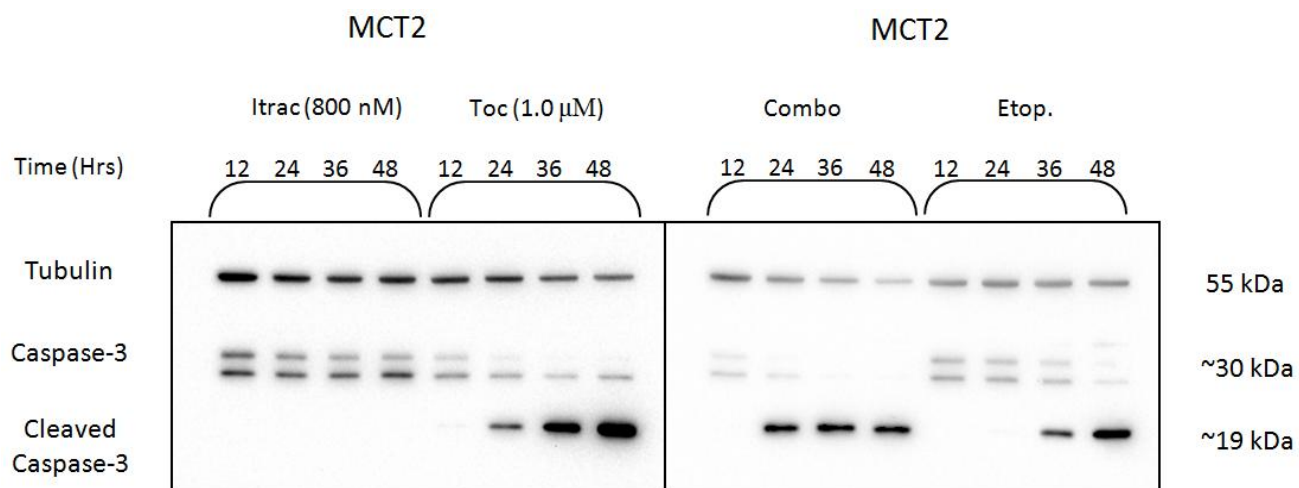


Figure 15. Western blots showing caspase activation in MCT2 cells in response to time course drug treatment. Four treatment groups (itraconazole, toceranib, combination itraconazole/toceranib and positive control etoposide) and four time points (12, 24, 36 and 48 hours) were used to determine caspase activation. N=3

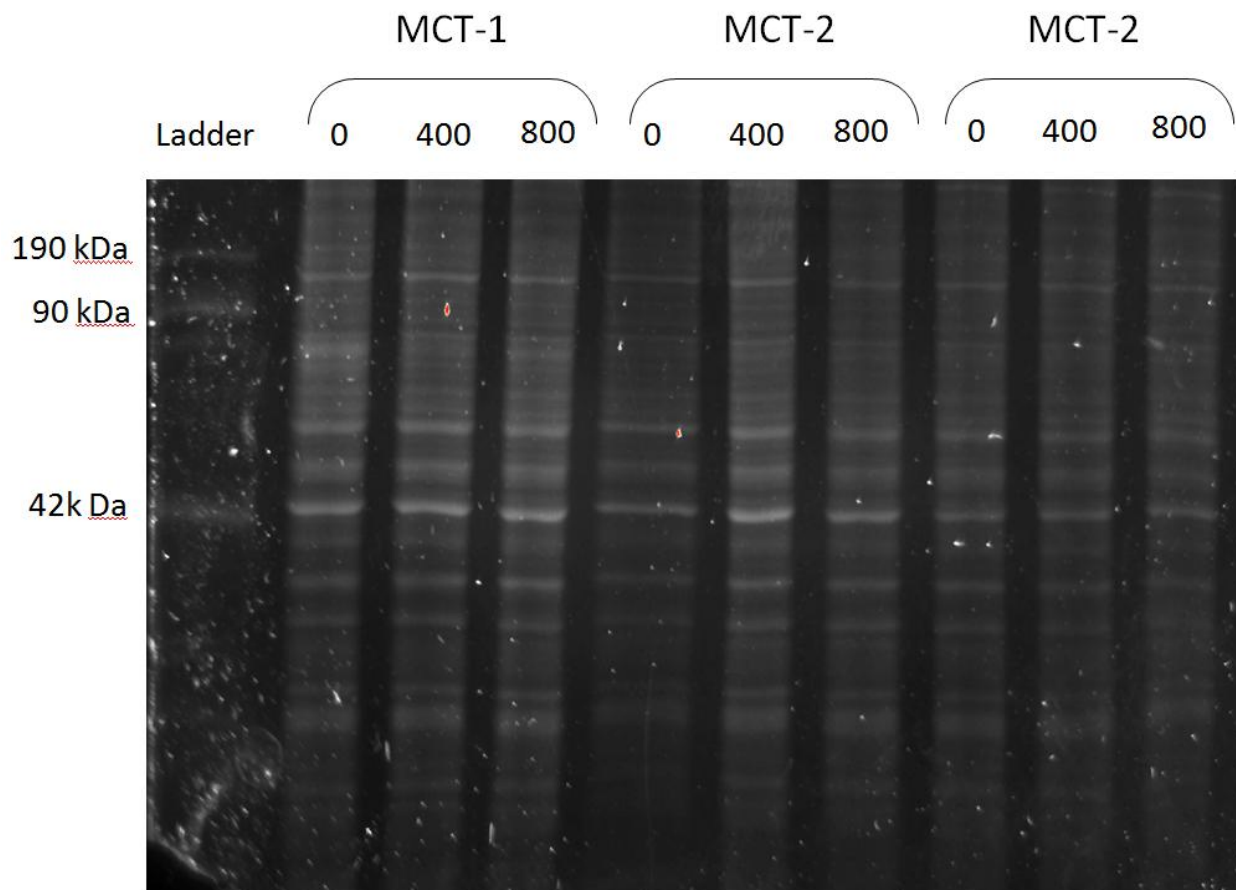


Figure 16. Acrylamide gel stained with ProQ Emerald 488 Glycoprotein dye. Each well was loaded with WCL isolated from MCT cells after 24 h treatment with 0, 400, or 800 nM of itraconazole. Protein was separated by gel electrophoresis, with stained bands representing glycosylated protein. Glycoprotein staining indicates no significant change in global protein glycosylation after 24 h of treatment. N=3

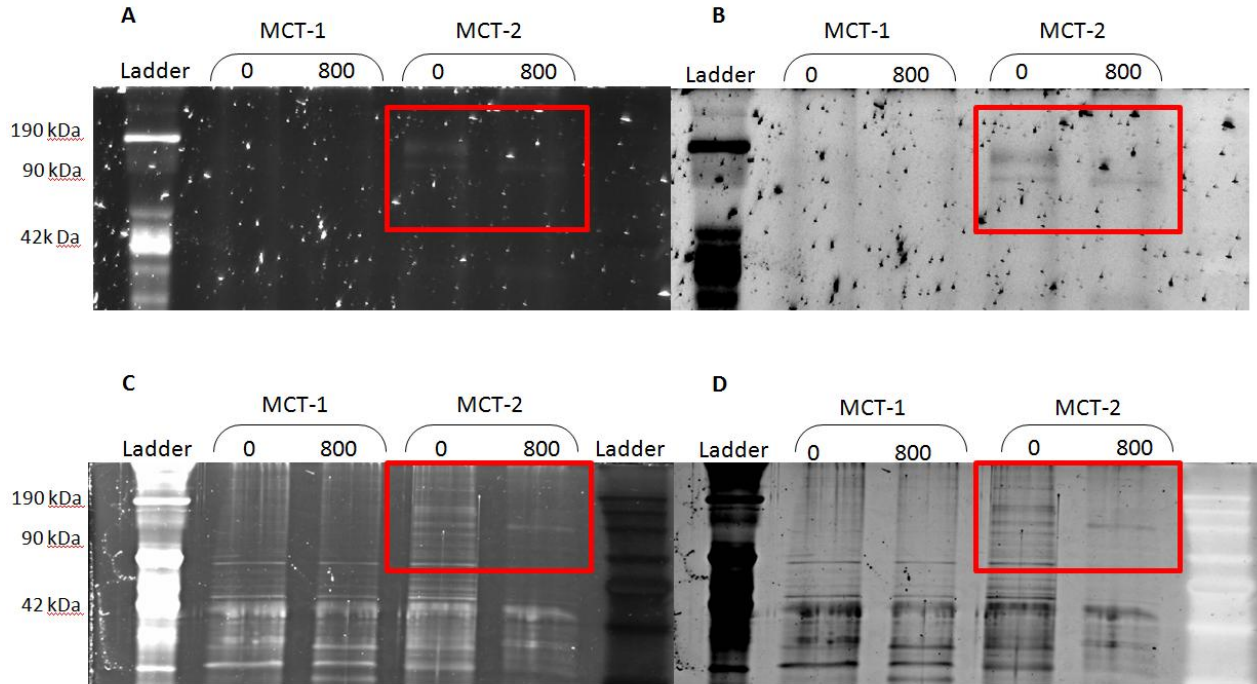


Figure 17. Glycosylation analysis of the KIT receptor using immunoprecipitation and Pro-Q Emerald 488 glycoprotein stain. Each well was loaded with immunoprecipitated KIT, extracted and purified from MCT WCL after 24 h treatment with 0 or 800 nM of itraconazole. (A) shows positive/normal image of acrylamide gel, (B) shows the same image in negative/reverse to enhance contrast, (C) shows the same acrylamide gel stained with SYPRO Ruby total protein stain to observe differences in total KIT protein, (D) shows the same image of the SYPRO ruby stain in negative/reverse to enhance contrast. Indicated within the red box in all images are the MCT2 lanes, which clearly show abolishment of KIT upper band. N=1

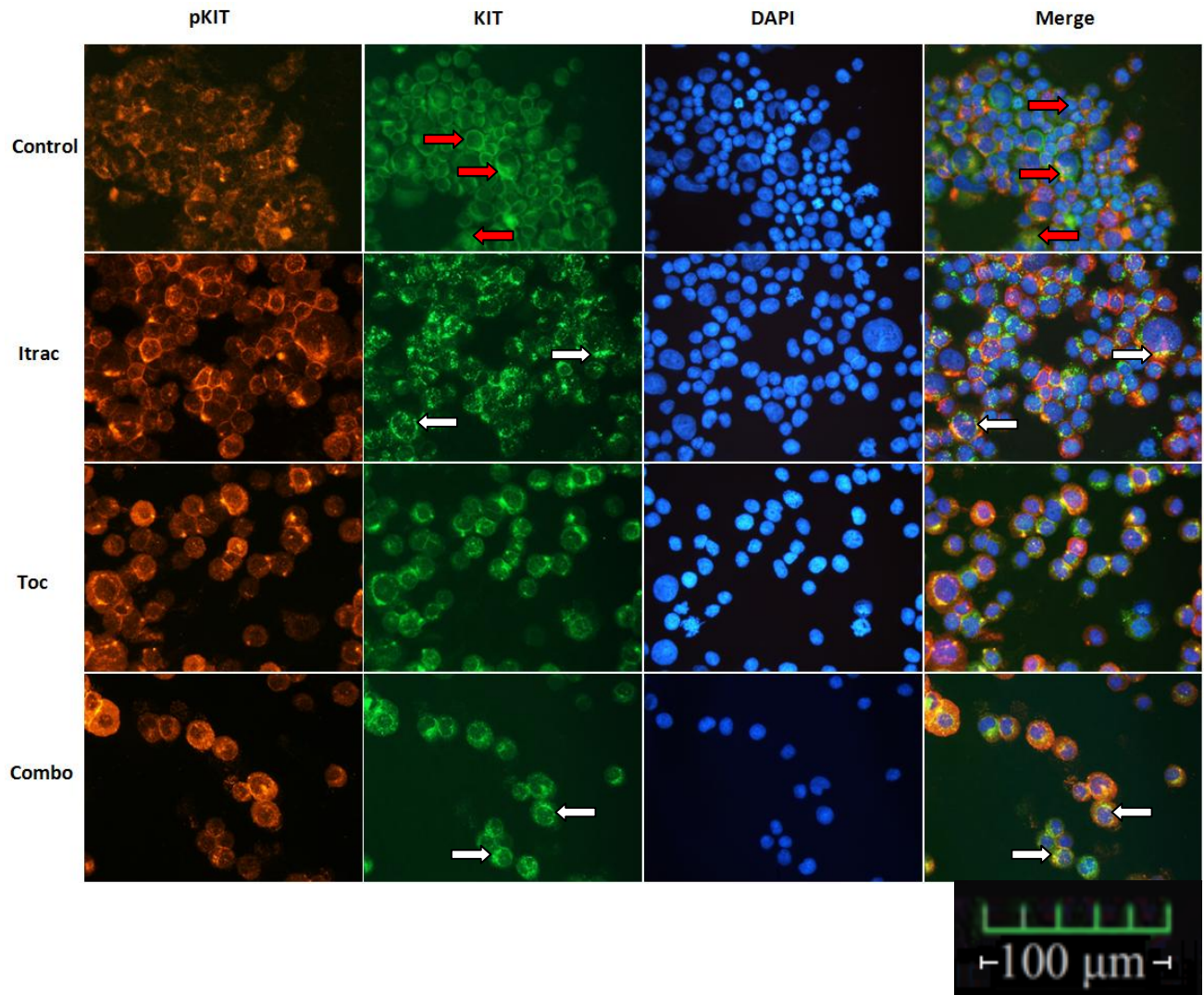


Figure 18. Immunofluorescence images of MCT1 cells after 24 hours of treatment. Four control groups of no drug control, 800 nM itraconazole, 1.0 μ M toceranib and combination of the two drug doses listed previously. Images show dual staining of pKIT and KIT, with DAPI nuclear stain showing nuclei and merge image showing overlay of all three stains. Red arrows indicate primarily perimembranous or diffuse cytosolic localization of KIT in untreated MCT1 cells and white arrows indicate primarily stippled cytoplasmic localization of KIT after itraconazole treatment.

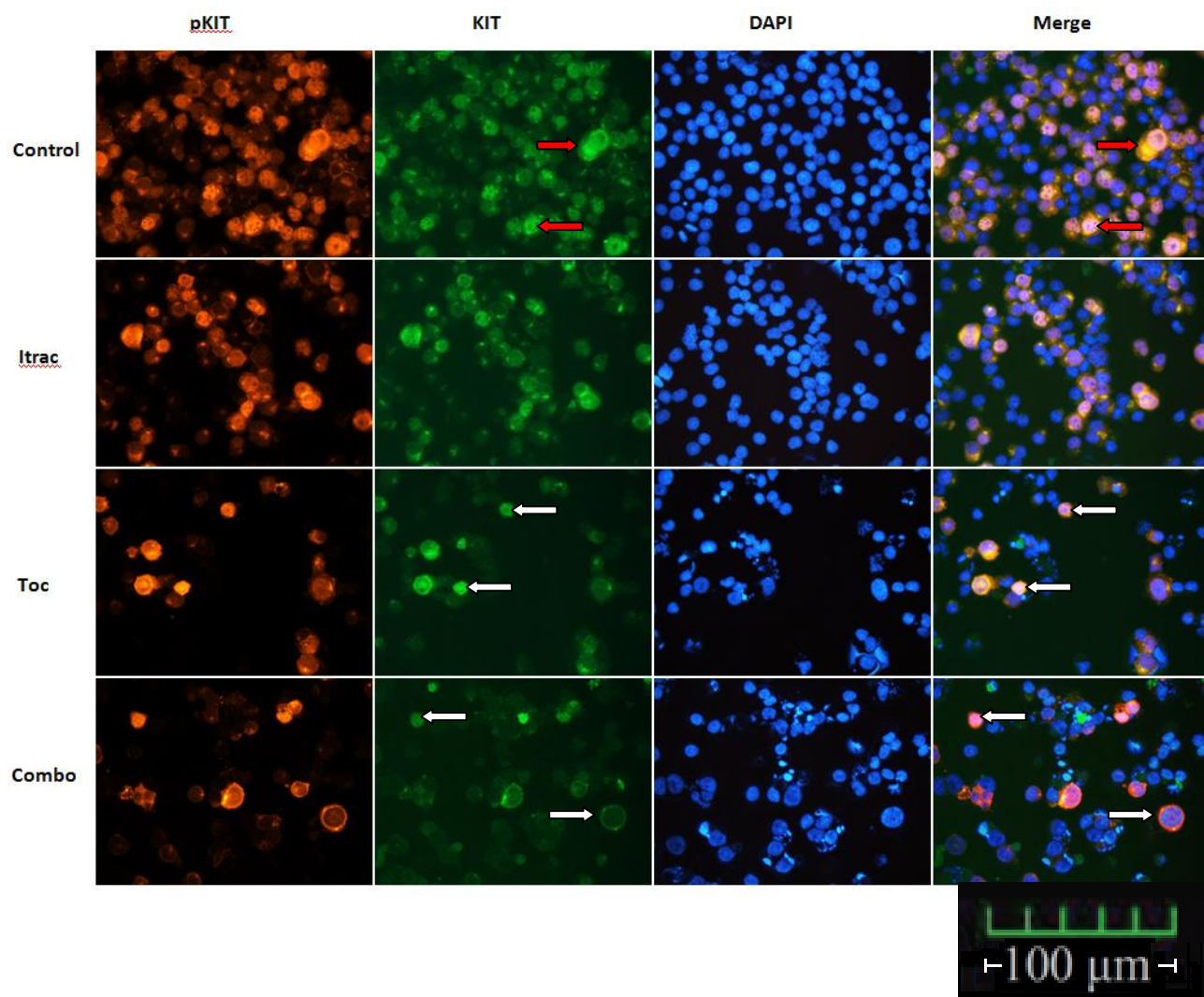


Figure 19. Immunofluorescence images of MCT2 cells after 24 hours of treatment. Four control groups of no drug control, 800 nM itraconazole, 1.0 μM toceranib and combination of the two drug doses listed previously. Images show dual staining of pKIT and KIT, with DAPI nuclear stain showing nuclei and merge image showing overlay of all three stains. Red arrows indicate primarily cytosolic localization of KIT in untreated MCT2 cells and white arrows indicate primarily nuclear or perimembranous localization of KIT after treatment.

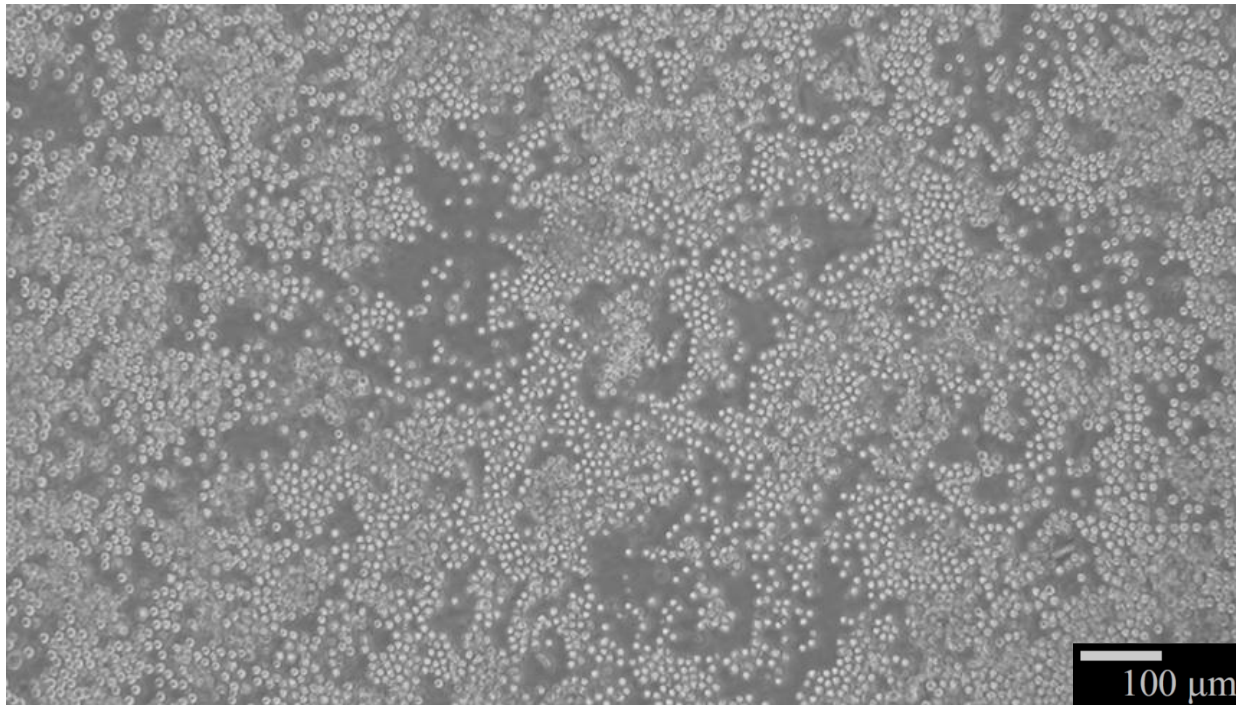


Figure 20. Microscope image showing novel MCT cells isolated from primary tumour sample. Neoplastic mast cells were isolated and purified from a tumour sample, with densely cellular populations persisting for weeks. Image was taken (under green illumination) approximately 5 days after isolation. Cells have survived several passages and have been cryopreserved. 400X magnification.

DISCUSSION

RTK research has become a critical component of developing novel, safe and more effective cancer treatments [12, 28]. Investigating the signalling properties and resistance mechanisms of RTKs at the cellular level is therefore essential to developing TKIs. Concurrently, it is critical for oncologists to investigate treatments that are also cost effective. This makes efficient treatment a viable option, available on a widespread basis for both humans and their pets. In this project, I have shown that a safe and inexpensive drug exhibits significant anti-cancer utility. This study is the first of which I am aware that shows itraconazole has anti-proliferative effects on canine mast cell tumour cells.

Mast cells are immune system cells critical for regulating allergic, inflammatory and immunological responses [1, 4]. Genetic mutations in mast cells lead to a number of human and canine diseases [64-66], with this project focusing on canine MCT. This disorder represents a serious economic and emotional burden for pet owners, with 7-21% of all canine skin tumours diagnosed as MCT [21, 70]. A significant proportion (often as high as 30%) of MCT cases also exhibit mutations in the *c-kit* gene, ultimately leading to the development of tumours driven by KIT dysregulation [7, 9, 17, 19]. It is consequently imperative to study this disease, not only to advance and improve veterinary oncology, but also to study KIT. Better understanding of KIT structure, signalling, mutations and especially drug resistance is needed to develop better prognostic indicators and treatments. This would be beneficial for treating numerous human disorders driven by KIT dysregulation, such as GIST, CML and NSCLC [116, 149, 155, 174].

The primary objective of my work was to use targeted therapy and canine MCT cells as a model for studying receptor inhibition and resistance. I first examined itraconazole, a widely

used and readily available anti-fungal drug recently found to possess anti-angiogenic activity [39]. This drug has a long history of safe application in both humans and dogs, and has such promise as an anti-cancer drug that it has been entered several human clinical trials [172-174]. Here I show that MCT2 cells are susceptible to itraconazole treatment, even at low doses (200 nM). Therapeutic drug monitoring studies have shown human patients with serum levels of itraconazole in excess of 1500 ng/mL after treatment periods for fungal infections, well above the concentrations used for this study [196]. This shows that even low doses of itraconazole can theoretically have significant anti-cancer efficacy, with higher doses easily achieved therapeutically. Anti-cancer effects at low doses could also significantly reduce drug costs and toxicity when compared to chemotherapeutics. During the course of my research, I showed that treatment for 24 hours results in the disruption of post-translational modifications of MCT2 cells (glycosylation and phosphorylation). Specifically, itraconazole induces a unique shift in the migration patterns of both KIT and VEGFR2. These receptors are critical for mast cell function, proliferation and survival [84, 88, 100-102].

My results clearly show that itraconazole treatment results in the downregulation of phosphorylation and disruption of receptor glycosylation after 24 and 48 hours of treatment, but are not observed before 8 hours. Both VEGFR2 and KIT typically appear as doublets on a western blot. The upper band represents mature and glycosylated protein of a higher molecular weight than lower band, non-glycosylated protein. As previously discussed, glycosylation is critical for proper protein folding and function [183, 184]. Improper glycosylation has been shown to lead to protease and chaperone activity that ultimately interferes with protein production, processing and shuttling [182-184]. The progressive downward shift and eventual disappearance of the upper band of both receptors indicates that itraconazole is completely

interfering with RTK glycosylation and hence signalling. Based upon my immunoprecipitation results, I would hypothesize that non-glycosylated RTK is degraded and recycled in treated cells. The absence of protein observed via total protein stains indicates that non-glycosylated RTK disappears due to itraconazole treatment. Although western blots generally show an upregulation of native, non-glycosylated protein after treatment, these blots show 24 hour treatment only. A 24 hour treatment period is likely not enough time to observe a significant degradation of non-glycosylated protein, hence explaining why the lower band does not exhibit downregulation. This conclusion would tally with protein quality control literature, which describes improperly glycosylated protein as generally compromised in their folding and signalling capabilities [182-184]. This leads to the effected protein being withheld and degraded; otherwise a build up of functionally useless protein would ensue. It is also important to note that changes in glycosylation generally take at least 16 hours to take effect [183, 184]. This would explain why 8 hour time course blots show no change in glycosylated KIT expression in response to itraconazole.

The results seen with itraconazole treatment are consistent with results seen by Nacev *et al.* [39]. They observed the same abolishment of the upper band of the VEGFR2 doublet in endothelial cells, and further investigation revealed significant downregulation of both VEGFR2 phosphorylation and activation of downstream signalling intermediates. Although the exact mechanism by which itraconazole interferes with glycosylation remains unknown, it appears that the effect is unique to itraconazole and not limited to specific species or cell types.

It is also evident that itraconazole selectively targets proteins, specifically RTKs. The western blots and immunoprecipitation experiments show that 24 hours of treatment is sufficient to disrupt the PTMs of KIT and VEGFR2. However, WCL glycosylation gels show that within

the same 24 hours of treatment there is no detectable change in the global protein glycosylation. This is a key finding, since it implies that itraconazole can potently and selectively target pathways important in MCT development. It is possible that itraconazole targets RTK subclasses due to their rapid turnover rate, especially if autocrine or paracrine signalling loops are present [197]. Endocytosis is a major form of regulating the signalling activity of RTKs [198, 199]. Ligand binding at the cell surface initiates rapid internalization of RTK-ligand complexes, which are then packaged into lysosomes to await degradation [199]. The loss of RTK due to the internalization process is typically offset by a greater rate of protein recycling and replacement [199]. This ensures there is always a maximal concentration of receptor at the cell surface to interact with ligand. However, itraconazole could be disrupting the delicate balance of internalization and recycling. If the glycosylation step of RTK production is disrupted by itraconazole, the proteins earmarked to replace internalized receptors would be likely be withheld and destroyed in the ER or the Golgi due to improper folding. Receptor levels at the cell surface would therefore not be replenished. This problem would be exacerbated in the presence of autocrine signalling loops, since constant and excess ligand production (SCF or VEGF) would quickly saturate the remaining RTKs at the cell surface. This would require rapid resupply of RTK, a task the cell would be incapable of completing due to interference by itraconazole. The overall consequence of itraconazole treatment would be a decline in RTK expression at the cell surface, and subsequent decrease in pathway activation.

This conclusion is supported by the limited data on itraconazole's anti-cancer effects, since both of these consequences were observed in endothelial cells treated with itraconazole [39]. The researchers found a decrease in VEGFR2 cell surface expression, and downstream signalling activation was significantly downregulated [39]. Ringhausen *et al.*, additionally found

that itraconazole treatment inhibited the trafficking of CD20 (an activated, glycosylated phosphoprotein) to the cell surface in human lymphocytes [200]. This further strengthens my hypothesis that itraconazole is selectively disrupting the production and trafficking of RTK in MCT cells.

There is an alternate hypothesis explaining disrupted RTK activity that would be particularly applicable if total RTK expression at the cell surface remained unchanged after itraconazole treatment. It is possible that receptors are in fact being successfully trafficked to the cell surface. However, the disrupted glycosylation process would affect the ability of the receptors to interact with ligand, dimerize and internalize. Glycosylation is important for receptor-ligand recognition [185] and subsequent dimerization and internalization [201]. Specific glycan residues can act as biochemical flags that are recognized by lectins on the ligand. The structural shape of the receptor, influenced largely by branched glycosylation, is also important for ligand interactions [201]. The importance of receptor glycosylation was illustrated by He *et al.*, who showed that mutant beta(1)-adrenergic receptors lacking typical N-glycosylation were unable to bind ligand, could not form dimers, and did not undergo ligand-promoted internalization [202]. It is therefore plausible that proteins with their glycosylation patterns disrupted by itraconazole could have difficulty in recognizing or binding their correlated ligand. This would lead to a decrease in pathway activation, despite total expression of cell surface receptor being unaffected.

Similar to most compounds that target RTKs, itraconazole undoubtedly does not target only KIT and VEGFR2. There will invariably be off target effects. Cells require a constant supply of new protein; hence it is probable that other significant pathways with high protein turnover rates will be affected by itraconazole treatment. This would likely explain the common

side effects of itraconazole, such as nausea, vomiting and diarrhea [211]. It is well established that cells lining the GI tract undergo rapid turnover due to the harsh environment. In such active cells, the demand for recycled protein is high. These cells would be particularly vulnerable to itraconazole treatment, and far more likely to experience cell death if protein production levels cannot meet demand. This hypothesis would explain why the previously listed side-effects are so common, since GI tract cells would be significantly effected by itraconazole treatment.

It is also important to note that itraconazole exhibits anti-cancer effects that appear unrelated to glycosylation disruption. This drug has been shown to potently inhibit the Hedgehog (Hh) signalling pathway. Hh signalling is critical for regulating tissue development during embryogenesis and stem and progenitor cell populations during adulthood [203, 204]. Hedgehog signalling is controlled by the transmembrane protein patched (PTCH), which inhibits the activity of Smoothened (SMO) [205]. When Hh ligands bind to PTCH, this inhibitory action is removed and SMO is activated. SMO recruits and activates transcription factors, which then translocate to the nucleus to regulate transcription of Hh target genes (such as GLI1, a gene critical for cell fate determination) [205]. Aberrant Hh signalling has been linked to oncogenic activity and maintenance of tumour progenitor cells in a variety of cancers, most notably medulloblastoma and basal cell carcinoma [206, 207]. However, itraconazole has been shown to inhibit Hh signalling by disrupting the activity of SMO. Itraconazole binds to SMO at a different site than other SMO inhibitors and suppresses activation, leading to significant anti-cancer effects [206]. In a Phase II trial of basal cell carcinoma patients, itraconazole was found to reduce cell proliferation by 45%, Hh pathway activity by 65% and tumour area by 24% [208]. Itraconazole has also been shown to be partially active against SMO mutants that are resistant to traditional SMO inhibitors [206]. Similar to previously reported anti-angiogenic effects, anti-Hh

effects are unique to itraconazole. Compounds in the same azole family did not induce any results similar to itraconazole [209].

The link between itraconazole's potent RTK inhibition and Hh suppression is not clear. It is plausible that itraconazole is disrupting the cellular and nuclear shuttling of SMO and Gli transcription factors, since glycosylation is important for such processes. However, this anti-SMO activity appears unrelated to protein glycosylation or RTK disruption [210]. Analysis of various itraconazole analogues revealed that strong inhibitors of Hh signalling did not potently disrupt VEGFR2 glycosylation. Concurrently, of the six strongest inhibitors of VEGFR2 glycosylation, only one strongly inhibited Hh signalling [210]. This information seems to indicate that itraconazole utilizes a variety of mechanisms to elicit anti-cancer effects. Although the details of these mechanisms remain unclear, a growing body of evidence continues to warrant further investigation of itraconazole.

The second major consequence of itraconazole treatment was a downregulation of phosphorylation of KIT and VEGFR2 in MCT2 cells. Changes in KIT phosphorylation mirrored the pattern shift of native KIT, with high molecular weight pKIT disappearing with 200 nM and higher dose treatment. Changes in pVEGFR2 were more variable, with upregulation observed at lower doses and downregulation observed only at highest dose of 800 nM. Despite this variability, the results indicate that itraconazole successfully interferes with the two major PTMs of RTKs, phosphorylation and glycosylation.

To further examine phosphorylation inhibition, I decided to use the TKI toceranib. Historically this drug has been shown to inhibit VEGFR, PDGFR and KIT signalling, leading to direct anti-tumour and anti-angiogenic activity against canine MCT and other cancers [158].

Time course experiments showed that toceranib potently inhibits KIT phosphorylation as early as 2 hours after initial treatment. This inhibition was evident in both cell lines, a significant result since previous work by London and Hanna showed that typically only canine MCT harbouring *c-kit* mutations (exon 11) were susceptible to toceranib treatment [193]. However, MCT1 (which has no detectable mutation in exon 11) displays the same downregulation as MCT2. The only apparent difference between cell lines was a slight recovery of KIT phosphorylation around the 8 hour mark in MCT2 cells. Interestingly, both cell lines experienced an upregulation of native KIT in response to toceranib treatment. The precise reason for this is unknown, but could possibly be due to accumulation of deactivated KIT receptor that has been internalized and awaiting degradation.

Next I sought to determine what effect disruption of RTK glycosylation and phosphorylation would have on cell growth. Cell number and viability assays clearly showed that itraconazole inhibited the proliferation of both cell lines at doses above 400 nM. Toceranib exhibited similar effects, with more noticeable drops in total cell number when compared to itraconazole treatment. The greatest drop in cell number for both cell lines was seen with triple combination itraconazole, masitinib and toceranib. However, the drug cocktail used to generate this data would likely be too toxic to be an appealing or therapeutically relevant option. The most promising treatment appeared to be combination itraconazole (800 nM) and toceranib (1.0 μ M). The likely reason being an effective twofold effect, whereby itraconazole disrupts receptor glycosylation and toceranib inhibits receptor phosphorylation. First, itraconazole alters or removes glycan residues on KIT and VEGFR2 through an unknown mechanism. Altering the glycosylation status prevents proper protein folding and trafficking [182-184]. This would likely lead to a decrease in RTK expression at the MCT cell surface, a result seen by Nacev *et al.*, when

treating HUVEC cells and macrophages with itraconazole [39]. Second, any mature receptor still at the cell surface or within the cell would have their ATP binding pockets competitively inhibited by toceranib. This leads to a decrease in overall levels of phosphorylated RTK, a result seen both in this study and others [39]. The consequence of disturbing RTK activity is the disruption of critical cell function and signalling pathways, ultimately inhibiting growth and proliferation. Here I have shown that itraconazole and toceranib, both singly and in combination, prevent the proliferation of two cell canine mast cell cancer lines, MCT1 and MCT2, *in vitro*.

To determine whether reductions in cell number were due to apoptosis, caspase expression was analyzed. MCT1 is clearly resistant to caspase-3 mediated apoptosis, as regardless of treatment group or time length, no significant caspase cleavage was observed. This means treatment by itraconazole and toceranib is unlikely to cause cell death in MCT1 cells. It is more likely that the cells are quiescent and have entered cytostasis. Cytostasis refers to the inhibition of cell growth and proliferation in response to some sort of stress [212]. The cells could also be senescent, where they have entered permanent growth and proliferation arrest but are still metabolically active to some degree [213]. Senescence can be triggered by telomere shortening or cellular stress. The presence of senescent MCT cells after treatment could be checked by observing morphology, since senescent cells often acquire an enlarged and flattened shape with increased granularity [213]. The expression of senescence-associated β -galactosidase could also be examined to determine if MCT1 cells are undergoing senescence [214].

The cells could also be undergoing cell death via another mechanism, such as caspase-9 mediated apoptosis or necrosis. Necrosis refers to the irreversible inflammatory cell death in response to metabolic or therapeutic stress [215]. These stresses induce a series of suicide signals in cancer cells that cause cell death. The presence of necrosis could be explored by quantifying

the expression of necrosis markers, such as poly (ADP-ribose) polymerase (PARP) [215]. Activation of PARP leads to release of nonhistone DNA-binding protein high-mobility group B1 (HMGB1) a protein loosely bound to chromatin that binds more tightly during apoptosis as the chromatin condenses [215]. High media levels of HMGB1 could therefore be used as an indicator of necrosis. Activation of the death receptors RIPK1 and TRAF2 could also be examined [215].

MCT2 cells however, are susceptible to caspase-3 mediated apoptosis. Although itraconazole does not seem to bring about cell death, toceranib appears effective at inducing apoptosis as early as 24 hours after treatment. Indeed, toceranib treatment induced caspase-3 cleavage more rapidly and effectively than etoposide, a positive control and known inducer of apoptosis [216]. This would explain why MCT2 populations experienced greater reduction in number compared to MCT1 cells. Toceranib treatment is likely inducing widespread cell death in this cell line, as evidenced by generation of cleaved caspase-3. The same general conclusion was also reached by a number of researchers who found MCT cell lines harbouring *c-kit* exon 11 mutations were sensitive to toceranib, with effects ranging from inhibition of growth and proliferation to significant induction of apoptosis [195, 217-219]. Accordingly, I feel combination toceranib and itraconazole treatment warrants further investigation in *in vivo* models to evaluate direct anti-tumour effects.

To confirm that itraconazole was in fact selectively affecting RTK glycosylation, I carried out glycosylation assays followed by immunoprecipitation of the KIT receptor. Cells treated with itraconazole exhibited no global change in glycosylation patterns. After 24 hours of high-dose treatment there is no change in the stained bands, indicating that no major glycan residues have been lost and the process of N-glycosylation has been largely uninterrupted.

However, I show that isolated and purified KIT receptor from MCT2 cells treated with the same high dose for 24 hours does exhibit changes in glycosylation patterns. The effect of itraconazole on receptor glycosylation is gaining more evidence. The latest studies show that itraconazole alters the glycosylation patterns on cell surface receptors in macrophages, disrupting their phagocytic abilities [220]. This evidence comes from follow up studies exploring previous work by Frey *et al.* [180]. Frey showed that itraconazole was interfering with complex N-linked glycosylation processing, leading to an accumulation of high-mannose glycoproteins on macrophage cell surfaces [180]. During the interval between these two studies, other researchers clearly demonstrated that itraconazole dramatically altered the glycosylation patterns of VEGFR2 in endothelial cells [39]. My research, along with the evidence from these studies, lends weight to my hypothesis and conclusion that itraconazole interferes with the glycosylation of RTKs that are critical for the survival of MCT cells.

I also used immunofluorescence to detect changes in KIT localization and phosphorylation in response to treatment. For MCT1, there is a noticeable shift in KIT localization from diffusely cytoplasmic to stippled and focal perinuclear staining. The shift from cytoplasmic to stippled localization could indicate that KIT is localizing to the golgi, where it is being retained after glycosylation disruption. Overall KIT expression in MCT2 was qualitatively downregulated, with diffuse cytoplasmic KIT staining largely abolished and replaced in part by perimembraneous localization. It is also essential to note the abundance of apoptotic bodies in the toceranib and combination treatments for MCT2. This corroborates data from western blots showing caspase cleavage and cell growth assays showing cell number reductions that toceranib induces cell death in MCT2 cells.

Finally, I have further shown that MCT cells can be isolated from primary tumour samples and maintained for a period of time in culture using previously described methods [191]. This method has advantages over others, since it has been shown to prolong longevity and viability of mast cells [191]. This process allows proper media replacement and waste removal, without significant loss of mast cells. While centrifugation is the standard practice for passaging cells, the acute fragility of mast cells renders centrifugation a nonviable option [186]. Furthermore, the co-culture of fibroblasts more accurately mimics a tumour microenvironment than monolayers. This creates cell lines that are likely more representative of true MCT behaviour than previous cell lines that have been passaged through mice.

Although I was unable to generate an immortalized cell line from numerous attempts, I have collected and cryopreserved several samples of viable MCT cells that show great promise for future attempts to establish a persistent population. If one of the cryopreserved samples can be maintained for prolonged periods into a novel cell line, they can be used to help overcome a barrier to studying canine MCTs by increasing our panel of available cell lines. The cell line could represent another valuable model for additional study of RTK signalling and inhibition. Most importantly, further cell lines would help build off my work by further examining KIT variability and resistance in canine MCT cells, thus providing better understanding of human disorders characterized by drug resistant KIT dysregulation.

Implications and Future Directions

The relatively new investigation into itraconazole has led to fascinating discoveries regarding the drug's effect on neoplastic cells. However, further research is required to fully elucidate itraconazole's role in potentially disrupting tumorigenesis for both human and canine disorders. In this investigation, I have used two canine MCT cell lines to examine itraconazole's effect *in vitro*. This was ultimately an attempt to supplement targeted RTK therapy by using a novel and inexpensive anti-cancer drug to disrupt RTK glycosylation. I have shown that itraconazole, both singly and in combination with TKIs, exhibits significant effects on receptor glycosylation. These data suggest that simultaneously targeting receptor glycosylation with itraconazole and receptor phosphorylation with TKIs can inhibit the proliferation of neoplastic cells, and in some cases induce cell death. However, more *in vitro* studies are required to further confirm the anti-proliferative effect itraconazole can have on cancer cells.

Using western blotting and immunoprecipitation, I have shown that KIT and VEGFR2 from MCT2 cells treated with itraconazole exhibit altered glycosylation patterns. I have further shown that glycosylation disruption leads to reduction in cell number, especially when combination TKI treatment is used. Additional work is required to corroborate this evidence. Flow cytometry could be carried out to determine the percentages of apoptotic, necrotic and healthy cells after itraconazole treatment. Quantifying the activation of downstream signalling intermediates via western blotting for both KIT and VEGFR2 controlled pathways is required to establish with certainty that signalling from these receptors is being inhibited.

While I have demonstrated itraconazole's effect on KIT glycosylation via immunoprecipitation, I was unable to replicate the results. This was most likely due to

inefficiencies in the protocol, possibly because of poor rates of antibody-bead coupling, antibody-KIT binding, or elution of KIT from the antibody-bead complex. Further attempts are necessary to confirm these results. IP of treated and untreated VEGFR2 is additionally needed to establish itraconazole's ability to target multiple RTKs. Exploring receptor glycosylation is also needed to establish the mechanism by which itraconazole disrupts the glycosylation process. Co-immunofluorescence localization studies should be carried out to determine where RTKs are localizing after treatment. This could discover if, and where (such as the ER or the Golgi), receptors are being withheld in the shuttling process. More in depth glycoanalysis is also required. Mass spectrometry could be used to analyze the glycan residues on RTK in detail, and compare treated and untreated receptors. These data could be combined with my findings to show how itraconazole interferes with glycosylation, and exactly what glycan residues are affected. Finally, long term itraconazole treatment studies should be carried out. Treating MCT cells with low dose itraconazole for several weeks followed by WCL glycosylation staining could discover if itraconazole has global effects on protein glycosylation. If no effects are found, it could establish itraconazole as a highly selective drug that targets RTKs typically responsible for tumorigenesis.

Prostaglandin E2 (PGE-2) could also be involved in MCT development, since it appears to induce human mast cells to secrete VEGF [8] and monocyte chemoattractant protein-1 (MCP-1), two critical pro-angiogenesis factors [139]. The levels of PGE-2 in canine MCT therefore warrant investigation, since it could be responsible for initiating pathological VEGFR activity

Although I have attempted to generate novel MCT cell lines, this effort has primarily enhanced the reputation of MCT cells being difficult to isolate and sustain in culture. No novel populations have yet been sustained into immortalized cell lines, but cryopreserved samples hold

promise for future attempts. It is essential to continue attempting to sustain neoplastic mast cells from primary tumour samples. Novel MCT cell lines are important models for studying RTK inhibition and resistance, two areas that are of critical importance for future cancer treatment.

Limitations

There were several limitations to this study. Although I have shown itraconazole has anti-proliferative effects on canine MCT cell lines *in vitro*, there is no evidence the drug will have the same effect on the complex tumour microenvironment found *in vivo*. It was also evident from cell number assays that combination therapy did not significantly result in additive or synergistic effects. This could mean that combining itraconazole with aggressive targeted therapeutics may not yield results worth the added drug toxicity.

My results clearly indicate itraconazole has an anti-proliferative effect on MCT1 and MCT2. MCT2 cell number assays and caspase-3 western blots reliably point towards apoptosis as responsible for initiating cell death in response to toceranib treatment. However, it is unclear what state MCT1 cells enter after itraconazole or toceranib treatment. Although proliferation is inhibited, it is unknown whether the cells are entering cytostasis or senescence. It must also be determined if there is a rescue effect, and whether the cells can re-enter the cell cycle after treatment is discontinued. This is a critical concern for cancer treatment, since the results would determine if patients would require prolonged itraconazole treatment to inhibit tumour growth.

I have shown altered KIT and VEGFR2 expression and migration patterns on SDS PAGE gels via western blotting in response to itraconazole treatment. While I have shown disruption of KIT glycosylation via IP experiments, I was unable to replicate these results. Due to time constraints and the failure of numerous attempts to duplicate the efficient IP of the KIT receptor that led to the positive result, I was unable to continue this experiment. Further work is needed to validate these results and confirm that itraconazole is interfering with the glycosylation process of RTKs.

Finally, it is also important to determine whether itraconazole elicits global effects on protein glycosylation. This study did not address the long term effects of itraconazole treatment, and how such treatment may be applied therapeutically.

SUMMARY AND CONCLUSIONS

The purpose of this study was to use canine MCT cells as a model for studying RTK signalling inhibition. In order to accomplish this goal, I sought to test a panel of TKIs and their anti-proliferative effects on two cells lines, MCT1 and MCT2. My primary focus however, was to examine the inhibitory potential of a novel and inexpensive anti-cancer drug called itraconazole. This led to my second objective of investigating itraconazole's ability to disrupt receptor glycosylation. Finally, this work addressed the glaring lack of available MCT cell lines by attempting to generate novel cell lines from primary tumour samples.

Both MCT cell lines exhibited a dose response effect to itraconazole treatment, with proliferation largely inhibited after treatment. In addition, both cell lines experienced reduction in cell number under high dose itraconazole (800 nM). The anti-proliferative effect on these cell lines is likely explained by RTK targeting, specifically itraconazole disrupting the activity of KIT and VEGFR2. Both of these receptors are critical for mast cell survival and proliferation, and their inhibition would likely have direct effects on the cell's ability to grow and divide.

The exact targeting mechanism used by itraconazole on RTKs is unknown, but disrupting receptor glycosylation is highly implicated. I have shown that both KIT and VEGFR2 exhibit altered native expression in MCT2 cells after itraconazole treatment. This effect is most likely due to an abolishment of high molecular weight species of KIT and VEGFR2, which represent the mature and glycosylated form of the receptors. I have further shown that KIT glycosylation is indeed being disrupted based upon immunoprecipitation and glycan staining. However, more work is needed to discover if VEGFR2 glycosylation is being similarly disrupted.

Inhibiting receptor activation was another primary area of this work. Both cell lines exhibited sensitivity to toceranib inhibition of KIT phosphorylation. Although additional validation is required to evaluate combination itraconazole and toceranib treatment, it would seem that simultaneously inhibiting glycosylation and phosphorylation of oncogenic RTK could elicit significant anti-tumour effects *in vivo*.

Validation could be found by generating novel MCT cell lines, another major component of this work. This would increase the available panel of cell lines, providing more models to studying RTK signalling, inhibition and resistance. It is also important to continue characterizing *c-kit* mutation status of cell lines and their associated susceptibility to targeted treatment. These types of models could be critical for advancing treatments for disorders such GIST, where a significant subset of patients (over 80%) harbour *c-kit* mutations [120], and would stand to benefit significantly from translational studies such as these.

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APPENDIX I – CHEMICAL LIST AND SUPPLIERS

0.5 M Tris-HCl Buffer pH 6.8	BioRad, Mississauga, ON, Canada
1.5 M Tris-HCl Buffer pH 8.8	BioRad, Mississauga, ON, Canada
4% Paraformaldehyde	USB Corporation, Cleveland, OH, USA
40% Acrylamide/Bis solution	BioRad, Mississauga, ON, Canada
8X Sample Buffer	Veterinary College, Guelph, ON, Canada
Amido Black	BioRad, Mississauga, ON, Canada
Aprotonin	Sigma-Aldrich, Oakville, ON, Canada
APS	BioRad, Mississauga, ON, Canada
Bio-Rad DC™ Protein Assay Kit	BioRad, Mississauga, ON, Canada
BLUeye prestained protein ladder	FroggaBio, Toronto, ON, Canada
BSA	Santa Cruz, Santa Cruz, CA, USA
Cell culture dishes	Fisher Scientific, Nepean, ON, Canada
Cell lysis buffer (10X)	Cell Signaling Technology, Danvers, MA, USA
Chemiluminescent substrate	Roche, Laval, Quebec, Canada
Collagenase type IV	Sigma-Aldrich, Oakville, ON, Canada
Coverlips	Fisher Scientific, Nepean, ON, Canada
Dako IF Pen	Dako, Carpinteria, CA, USA
Dako Protein Free Serum Block	Dako, Carpinteria, CA, USA
DAPI	Life Technologies, Burlington, ON, Canada
Dimethyl sulfoxide (DMSO)	Fisher Scientific, Nepean, ON, Canada
Dynabeads Antibody Coupling Kit	Life Technologies, Burlington, ON, Canada
Dynabeads Co-Immunoprecipitation Kit	Life Technologies, Burlington, ON, Canada
Ethanol	Fisher Scientific, Nepean, ON, Canada
FBS	Life Sciences, Burlington, ON, Canada
Filters (70 µM)	Millipore, Temecula, CA, USA
Fluorescent mounting medium	Dako, Carpinteria, CA, USA
Gentamicin	Sigma-Aldrich, Oakville, ON, Canada
Glycine	Fisher Scientific, Nepean, ON, Canada
HRP-labeled goat anti-mouse (A4416)	Sigma-Aldrich, Oakville, ON, Canada
HRP-labeled goat-anti-rabbit (A0545)	Sigma-Aldrich, Oakville, ON, Canada
Hydrogen Peroxide, 30%	Fisher Scientific, Nepean, ON, Canada
Itraconazole	Millipore, Temecula, CA, USA
Luminata™ Forte	EMD Millipore, Darmstadt, Germany
Lysis buffer	Cell Signaling Technology, Danvers, MA, USA
Masitinib	AdooQ Bioscience, Burlington, ON, Canada
Methanol	Fisher Scientific, Nepean, ON, Canada

Mouse-anti-tubulin antibody (T9026)	Sigma-Aldrich, Oakville, ON, Canada
Mouse-anti-cKIT mAb Alexa Fluor 488 conjugate antibody (3310S)	Cell Signaling Technology, Danvers, MA, USA
MTT-based Cell Growth kit	Sigma-Aldrich, Oakville, ON, Canada
Normal Goat Serum	Life Technologies, Burlington, ON, Canada
Paraformaldehyde, 4%	USB Corporation, Cleveland, OH, USA
PBS	Sigma-Aldrich, Oakville, ON, Canada
Penicillin/Streptomycin	Lonza Group, Mississauga, ON, Canada
Phenol-Red Free RPMI 1640	Sigma-Aldrich, Oakville, ON, Canada
PMSF	Sigma-Aldrich, Oakville, ON, Canada
Poly-L-Lysine high adhesion cell culture plates	Becton Dickison, Bedford, MA, USA
Protease inhibitor cocktail II	Sigma-Aldrich, Oakville, ON, Canada
ProQ Emerald Glycoprotein Kit	Life Technologies, Burlington, ON, Canada
PVDF membrane	Roche, Laval, Oakville, ON, Canada
Rabbit-anti-cKIT antibody (A4502)	Dako, Carpinteria, CA, USA
Rabbit-anti-phospho-cKIT antibody (3391S)	Cell Signaling Technology, Danvers, MA, USA
Rabbit-anti-phospho-VEGFR-2 antibody (2471S)	Cell Signaling Technology, Danvers, MA, USA
Rabbit-anti-VEGFR-2 antibody (2472S)	Cell Signaling Technology, Danvers, MA, USA
Recombinant Canine Stem Cell Factor (rSCF)	R&D Systems, Minneapolis, MN, USA
RPMI 1640	Sigma-Aldrich, Oakville, ON, Canada
RPMI 1640 without phenol red or L-glutamate media	Sigma-Aldrich, Oakville, ON, Canada
SDS	BioRad, Mississauga, ON, Canada
Sodium pyruvate	Sigma-Aldrich, Oakville, ON, Canada
Sodium chloride	Fisher Scientific, Nepean, ON, Canada
Sodium dodecyl sulfate (SDS) 20%	Fisher Scientific, Nepean, ON, Canada
Sodium orthovanadate	Fisher Scientific, Nepean, ON, Canada
Stripping solution, Reblot Mild	Millipore, Temecula, CA, USA
Sypro Ruby total protein stain	Life Technologies, Burlington, ON, Canada
TEMED	Roche, Mississauga, ON, Canada
Toceranib	Pfizer Inc.
Tris Base	Fisher Scientific, Nepean, ON, Canada
Triton X-100	BioRad, Mississauga, ON, Canada
Trypan blue 4%	Sigma-Aldrich, Oakville, ON, Canada
Trypsin	Veterinary College, Guelph, ON, Canada
Tween 20	Fisher Scientific, Nepean, ON, Canada

APPENDIX II – PREPARATION OF SOLUTIONS

12% Resolving Gel (1.5 mm Gel)

4.35 ml MQ H₂O

3 ml 40% acrylamide-bis solution

2.50 ml 1.5 M Tris-HCl (pH 8.8)

100 µl 10% SDS

120 µl 10% APS

5 µl TEMED

Mix all components together and immediately add to space between plates. Top off with isopropanol to prevent uneven polymerization.

Once polymerized (~20 minutes), pour off the isopropanol and add the stacking Gel.

7.5% Resolving Gel (1.5 mm Gel)

5.49 ml MQ H₂O

1.86 ml 40% acrylamide-bis solution

2.50 ml 1.5 M Tris-HCl (pH 8.8)

100 µl 10% SDS

120 µl 10% APS

5 µl TEMED

Mix all components together and immediately add to space between plates. Top off with isopropanol to prevent uneven polymerization.

Once polymerized (~20 minutes), pour off the isopropanol and add the stacking Gel.

5% Stacking Gel (1.5 mm Gel)

1.59 ml MQ H₂O

250 µl 40% acrylamide-bis solution

630 µl 1.5 M Tris-HCl (pH 6.8)

25 µl 10% SDS

25 µl 10% APS

2.5 µl TEMED

Mix all components together and immediately add space between plates. Add a comb and allow to polymerize.

10X Electrophoresis Buffer

144.2 g Glycine

30.2 g Tris base

50 ml 20% SDS

Dissolve Tris and glycine in MQ H₂O to a volume of ~850 mL, adjust to a pH of 8.3 using HCL and top up with MQ to a final volume of 1 L.

Store at room temperature. Dilute to 1X in MQ H₂O when needed.

Towbin's Solution

144.1 g Glycine

30.25 g Tris base

Dissolve in MQ H₂O to a final volume of 1 L. Store at room temperature.

Wet Transfer Buffer

720 ml MQ H₂O

200 mL Methanol

80 mL Towbin's Solution

200 µl 2% SDS

Mix all components and store at 4°C until needed

10X TBS

24.25 g Tris base

80.0 g NaCl

Dissolve in MQ H₂O to a final volume of ~850 mL, adjust to a pH of 7.6 using HCL and top up with MQ H₂O to final volume of 1 L.

1X TBS-T

900 mL MQ H₂O

100 mL 1X TBS

1.0 mL Tween 20

Mix well to ensure Tween 20 is completely dissolved.

Semi-dry Transfer Buffer

5.82 g Tris-base

2.93 g Glycine

3.75 mL 10% SDS

200 mL Methanol

Dissolve in MQ H₂O to final volume of 1 L

Sodium Orthovanadate Solution

Per 1 mL of culture solution;

33.25 µL PBS

2.5 µL 3% H₂O₂

6.25 µL Sodium Orthovanadate