RECEPTOR TYROSINE KINASE TARGET VALIDATION AND COMBINATORIAL STRATEGIES TO CIRCUMVENT SMALL MOLECULE THERAPEUTIC RESISTANCE IN CANINE METASTATIC INSULINOMA

BY

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THESIS

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ABSTRACT

Canine insulinoma is a malignant and metastatic tumor of the endocrine pancreas. Clinical evidence suggests that toceranib phosphate (Palladia), a small molecule receptor tyrosine kinase (RTK) inhibitor of c-kit, VEGFR, and PDGFR, might improve outcomes in dogs with insulinoma; yet some affected dogs do not derive benefit. Surprisingly, validating the expression and functionality of toceranib molecular targets has not been confirmed in canine insulinoma, nor have combinatorial strategies explored for overcoming resistance mechanisms. Provocatively, Phase I human trials have identified PAC-1, a small molecule activator of procaspase-3 (PC3), to exert not only single-agent activity in neuroendocrine tumors, but also to circumvent resistance mechanisms employed by cancer in response to RTK inhibitors. Herein, we determine if molecular targets of toceranib and PAC-1 are expressed in canine insulinoma, and if combination treatments can generate synergistic killing and unique gene transcriptional responses, along with how they affect survival and proliferation pathways. A canine insulinoma cell line (canINS), outcome-linked insulinoma tissue microarray, and 20 canine paired primary and metastatic insulinoma tissues were evaluated for PC3, c-kit, VEGFR, and PDGFR expression. Sensitivity and transcriptional responses of canINS to toceranib and PAC-1 were evaluated via viability assays, western blot, and RNAseq. Insulinomas express c-kit and VEGFR, but not PDGFRβ in meaningful amounts. Expression of PC3 was identified in most samples, with expressions being highest in metastatic lesions and correlated with prognosis. In culture, canINS

was resistant to Palladia alone, but combination with PAC-1 resulted in sensitization. RNAseq identified putative drug resistance responses and unique cell death induction pathways. Members of the MAPK and AKT pathway were affected by treatments. Canine insulinomas express molecular targets that are druggable with oral therapeutics (toceranib/PAC-1), and when combined, exert synergistic anticancer activities. Given these additive properties, toceranib and PAC-1 would be predicted to improve treatment responses in dogs with metastatic insulinoma.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

CHAPTER 1

Introduction

Pancreatic beta cell neoplasia is the most common neuroendocrine cancer of the canine

pancreas. The disease is hallmarked by an over-production of insulin, leading to the colloquial

term "insulinoma". Insulinomas have a relatively high metastatic rate with fifty percent of dogs

diagnosed with metastatic lesions at the time of diagnosis, predominantly to the liver and local

lymph nodes. Ideal practice is surgical removal of all lesions, often precluded by extent of local

and/or metastatic disease. Surgery is not considered curative and recurrent local or distant

disease from micro-metastases at the time of surgery is common with a median survival time of

around eighteen months following surgical intervention. Traditional adjuvant chemotherapy

has not been found to provide meaningful extension of survival times. Attempts at cytotoxicity

of the primary disease have come with unacceptable adverse effects, such as the case with

streptozocin. Attempts at disease control are therefore largely palliative, with the goal of

ameliorating effects of hyperinsulinemia and subsequent hypoglycemia. While this can improve

quality of life and temper neuroglycopenia for some time, treatments such as octreotide,

diazoxide, and prednisone do not target the cancerous burden. New strategies are therefore

warranted.

Functional pancreatic neuroendocrine tumors occur in humans and surgery is also the

primary form of treatment. Meaningful therapeutics for advanced pancreatic neuroendocrine

tumors (PNETs) are lacking, however the small molecule tyrosine kinase inhibitor sunitinib has

recently shown promise in this context. In initial phase I and II trials, sunitinib demonstrated

antitumor activity in patients with pancreatic neuroendocrine tumors. In a phase III trial focusing on sunitinib in the context of PNETs, progression free survival was significantly prolonged compared to the placebo group. Toceranib phosphate is a veterinary small molecule inhibitor with remarkable molecular similarity to sunitinib. There are limited studies in veterinary medicine assessing efficacy of toceranib in cases of canine insulinoma, however the studies that have been performed suggest treatment to be beneficial. While sunitinib exhibited significant differences in survival in cases of human PNETs, overall response rate was only about 9%. Response rates of canine insulinoma to toceranib were found to be higher in the veterinary literature, however these studies were retrospective in nature and included various concurrent treatment modalities, precluding accurate analysis of these results. Multimodal therapy for advanced disease is strategically warranted. Combinations of therapeutics can be beneficial in their additive effects; however it is also feasible for drugs to act synergistically with one another.

Procaspase activating compound 1 (PAC-1) is a small molecule that has been studied in the context of synergism with traditional oncologic therapeutics, along with as a single agent. PAC-1 functions through chelation of zinc molecules that exert inhibition on the activity of procaspase(s), most notably procaspase-3 (PC-3), thereby biasing a cell towards apoptosis. PC-3 has been found to be overexpressed in a variety of tumor types, leveraging itself as an attractive target for therapy. PAC-1 has shown synergism with chemotherapy treatments in pet dogs, and is found to be safe when given to these patients.

In this investigation, our goal was to determine if toceranib and PAC-1 combination treatment is a reasonable option for pet dogs with malignant insulinoma. We aimed to identify the expression of the targets of both toceranib and PAC-1 in spontaneous canine insulinomas, and to identify their use in targeting these cancerous cells in vitro. We also aimed to investigate transcriptional and post-transcriptional modifications that occur in these cells as a result of exposure to these drugs. We hypothesize that combining toceranib and PAC-1 will improve treatment outcomes for pet dogs with insulinomas.

Chapter 2

Literature Review

2.1 Insulinoma biology

2.1.1 Clinical background and treatment options

Insulinomas are the most common neuroendocrine tumor of the canine pancreas and are hallmarked by an over-production of insulin by neoplastic beta cells that are non-responsive to typical glucose homeostatic mechanisms. Insulinomas develop from malignant transformation of pancreatic beta cells, along with which the other cells of the endocrine pancreas reside within Islets of Langerhans. Beta cells make up around 70% of these islets (1).

The etiology of insulinomas is not currently known. A transcriptomic study revealed over 3,000 differentially expressed genes between pancreatic beta cells and insulinoma cells, with upregulation of genes involved in beta cell fate, along with alpha and ductal cell genes (2). There has been additional evidence to suggest that pancreatic islet cell tumors may arise from acinar/ductal tissue rather than directly from islet tissue (2,3). Normal pancreatic tissue expresses growth hormone weakly, and neoplastic tissue has been shown to express it in higher amounts with the most being produced by metastatic disease compared to the primary tumor (4). This may be significant as growth hormone has been shown to be mitogenic for beta cells via the Jak/STAT pathway (5,6). Additional growth factors shown to have mitogenic effects on pancreatic beta cells include other lactogenic hormones, hepatic growth factor, PTHrp, insulin

and insulin-like growth factors and glucagon-like peptide-1 and local production of these hormones may have autocrine or paracrine effects (5).

Due to a lack of knowledge regarding what causes these tumors to arise, treatment is reactive, and primarily focused on the local disease in addition to managing subsequent hypoglycemia. In dogs with locoregional disease amenable to resection, surgery via partial pancreatectomy is the recommended treatment option. Dogs whose disease can be reduced to microscopic disease burdens by surgery alone accomplish a median survival time of 12-18 months. Potential post-operative complications include development of transient hyperglycemia, diabetes mellitus, and pancreatitis (6).

For many patients, surgery is not feasible or recommended due to disseminated disease, as 50% of dogs with insulinomas present with metastatic disease predominantly to local lymph nodes and the liver. For those with locoregional disease only, surgery is still recommended. There are medical options for those dogs where surgery is not recommended with varying degrees of success and potential adverse effects. Additionally, the majority of medical options target glucose handling solely, and do not address the neoplastic cells themselves. Typical management strategies employ exercise restriction and diet modification with small, frequent meals to maintain steady states of glucose in the blood. Corticosteroid therapy is often used to antagonize the activity of insulin and to stimulate hepatic gluconeogenesis. Early studies into medical management found this strategy to be largely inferior to surgical excision, but significantly useful in the treatment of nonresectable or relapsed disease (7,8). Pharmacologic strategies to decrease the production or release of insulin

by pancreatic beta cells include drugs like diazoxide and octreotide. There is a reported response rate of 50-70% for these drugs, and their use can be limited by cost, availability, and development of resistance (9,10,11,12,13). Diazoxide is a thiazide drug originally marketed for treating hypertension. It was found to be diabetogenic in humans, as it blocks a sulfonylurea receptor on pancreatic beta cells. Blocking this receptor increases the beta cells' permeability to potassium resulting in hyperpolarization and inhibition of calcium-dependent insulin release (14). Octreotide is a somatostatin analogue, which can bind to the G-protein coupled somatostatin receptors type 5 on pancreatic beta cells. These receptors are coupled to the inward rectifying potassium channels, and activation leads to inhibition of the activation of voltage-gated calcium channels. This reduces cAMP levels and inhibition of the release of insulin (15). While studies have shown insulin levels to be significantly reduced when using octreotide in cases of canine insulinoma, glucose levels were not significantly affected, and therefore clinical use is questionable (16,17).

Streptozocin is a nitrosurea alkylating chemotherapeutic agent that provides a directed therapy towards neoplastic beta cells. The drug enters pancreatic beta cells via the highly expressed GLUT2 transporter and causes alkylation of the cellular DNA, which is ultimately cytotoxic (18). It was first found to increase blood glucose in a dog with insulinoma in the 1970's. This dog also developed a nephropathy via defects to the renal tubules, along with liver insult (19). Streptozocin toxicity to the renal tubules has also been shown to develop in humans, and induction of diuresis via sodium chloride fluid administration has been reported to help prevent the renal toxic effects in humans, and this was shown to also be the case in dogs

(20). Some dogs have shown measurable reductions in tumor size with streptozocin treatment,

however durable effects on blood glucose have not been demonstrated (20,21).

The prognosis for canine insulinoma is dependent on tumor size and stage. With stage I

or II disease that can be decreased to microscopic burden with surgery, median survival times

are around 1.5 years. Regarding glycemia, half of dogs with stage I disease will be not

hypoglycemic at 14 months following surgery. This is compared to 20% with stage II or III. For

dogs with stage II or III disease that is not amenable to surgery, survival times approach 6

months (10).

2.1.2 <u>Glucose handling</u>

Pancreatic beta cells are the sole producers of the hormone insulin. Insulin is a small

protein, initially produced as a preprohormone in the endoplasmic reticulum of beta cells, then

cleaved to proinsulin, and finally cleaved in the Golgi apparatus into insulin. This final cleavage

produces the insulin hormone along with the cleaved connecting peptide, termed the C

peptide. Insulin and the C peptide are packaged together and released from the beta cells via

exocytosis of secretory granules. The C peptide has no insulin activity (Fig 1).

The stimulus for secretion of insulin from the beta cells is glucose in the blood. Beta cells

contain within their membrane numerous GLUT2 glucose transporters that allow independent

glucose influx. The degree of influx into the beta cells is directly proportional to the blood

concentration of glucose. Similar to the way in which the majority of somatic cells handle

intracellular glucose, the glucose is readily phosphorylated within the beta cells by glucokinase

to produce glucose-6-phosphate. This step appears to be the primary way in which beta cells

sense blood glucose. The glucose-6-phosphate is then oxidized, leading to the production of ATP. Membranous potassium channels sensitive to ATP close with the increased amounts of ATP leading to cellular depolarization and subsequent opening of voltage-gated calcium channels with calcium influx. This calcium stimulates the exocytosis of insulin-containing secretory vesicles. Some hormones are able to cause an increase in intracellular calcium within the beta cells and are able to enhance the insulin-releasing effects of glucose, however do not have significant effects on insulin secretion without glucose. Counterregulatory hormones, secreted in response to hypoglycemia, act to inhibit secretion of insulin. Overall, the glucose blood concentration is the primary regulator of insulin secretion (1).

In addition to stimulating secretion of insulin, glucose presence within the beta cell also increases the synthesis of insulin by the beta cells. Glucose stimulates the recruitment of the transcription factors PDX-1, MafA, and B2 to increase expression of the insulin gene. Glucose also leads to increased stability of the preproinsulin mRNA (1).

Neoplastic pancreatic beta cells fail to respond appropriately to low blood glucose signaling. Genetic control of non-neoplastic pancreatic beta cells has been well-characterized and insulin- associated genetics have been found to be highly regulated and under precise control. This is in contrast to neoplastic beta cells whose insulin production continues unabated in the context of hypoglycemia. Glucokinase is an isoenzyme of hexokinase, expressed specifically in the endocrine pancreas and liver. Glucokinase is the major glucose-sensor in these cell types, providing translation between extracellular glucose concentration and the rate of intra-cellular glucose phosphorylation. Overexpression of glucokinase modulates glucose

responsiveness in vitro, and a study that induced overexpression of glucokinase in pancreatic beta cells led these cells to become hypersensitive to glucose with an increased rate of glycolysis and subsequent insulin release (11). Molecular studies of canine and feline insulinoma have suggested abnormalities in glucokinase expression (12). Cultured insulinoma cells from two dogs demonstrated increased glucokinase expression compared to normal pancreatic tissue from the same two dogs (11). Molecular characterization of a feline insulinoma demonstrated a 17-fold increase in glucokinase transcripts compared with adjacent normal pancreas (22). Increased expression of glucokinases may represent a mechanism by which these cells are stimulated to over-produce insulin in vivo. Additionally, neoplastic beta cells are still responsive to elevations in glucose, and often have an exaggerated response (23,24).

2.2 Tyrosine kinase inhibitors

2.2.1 Mechanism of action

Receptor tyrosine kinases are cell surface receptors that transmit extracellular signals to the nucleus of the cell via serial phosphorylation of signal transduction proteins (Fig 2). These receptors consist of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic region with a juxtamembrane domain, C-terminal tail, and tyrosine kinase domain. The tyrosine kinase domain contains a small N-terminal (N) lobe and a C-terminal (C) lobe, and includes an activation loop, of which the orientation determines the active or inactive state. The extracellular binding domains can exist as a monomer, dimer, or oligomer. The majority of extracellular binding domains exist as monomers, and ligand binding (a growth factor or cytokine) mediates dimerization of two receptor monomers. Dimerization induces rotation of

the N and C lobes, and the activation loop moves. A tyrosine molecule is present in the kinase

active site blocking ATP binding. Ligand dimerization permits the partner tyrosine kinase

domain to transphosphorylate the inhibitory tyrosine molecule, thereby releasing it from the

active site and allowing ATP to bind. As a consequence to transphosphorylation, the receptor

molecule displays a spectrum of phosphotyrosine molecules on its cytoplasmic tail. These

phosphotyrosines become homing sites for specific Src Homology 2 (SH2) sites of various

proteins. These proteins then elicit downstream cascades. For example, one possible protein

that becomes tethered to an RTK is Growth Factor Receptor Bound Protein 2 (Grb2). Its role is

to serve as an anchor for an additional protein called Son of Sevenless (SOS) which acts as a

guanine nucleotide exchange factor and causes GDP-bound RAS to become associated with GTP

and become activated. Activated Ras lies upstream of multiple signaling pathways that utilize

phosphorylation of serine and threonine residues on downstream kinases to mediate signal

through the cytoplasm to the nucleus. These pathways ultimately culminate on improved

growth, survival, and proliferation. While there are 58 unique known receptor tyrosine kinases,

there is redundancy in the signaling pathways that are used, with significant cross-talk present

(25).

2.2.2 Receptor tyrosine kinases and signal transduction pathways in cancer

Over-activity of receptor tyrosine kinases leads to increased nuclear expression of

proteins responsible for cell proliferation and survival (Fig 3). Constitutive activation of portions

of the receptor itself, or of members of the downstream signaling transduction pathways, is

oncogenic. Gene duplications of receptors or over-expression of these receptors can also lead

to increased activity and signaling of these pathways with the same oncogenic outcomes.

Autocrine loops in which cancer cells themselves produce the ligands for these receptor tyrosine kinases can lead to over-stimulation. There can also be inactivity of negative input onto these pathways, for instance the AKT/mTOR pathway, which is responsible for stimulation of protein synthesis, proliferation, and inhibition of apoptosis, is negatively impacted by the activity of phosphatase and tensin homolog (PTEN). Dysregulation of PTEN activity can also lead to increased activity of the AKT/mTOR pathway leading to inhibition of apoptosis and cellular proliferation. PTEN activity is lost in 30-40% of human cancers. Regardless of where the pathways are dysregulated, the final consequence is the disruption of the cell growth/survival and cell death (26,27).

The first major cell signaling pathway is the MAPK pathway (Fig 4). Following activation of Ras, Raf becomes phosphorylated by Ras, subsequently leading to phosphorylation and activation of MEK, and then ERK 1 and 2. Targets of ERK are located in both the cytoplasm and the nucleus, and these predominantly play a role in upregulation of genes within the cell cycle (25).

The second major pathway is the AKT pathway, which involves phosphatidyl inositol 3 kinase (PI3K) (Fig 5). PI3K gets recruited to the phosphorylated RTK via binding of the p85 adaptor subunit. This leads to activation of a catalytic subunit p110. This results in the generation of PIP3, which recruits AKT to the membrane. AKT has many downstream targets that are either activated or inhibited via phosphorylation by activated AKT. This pathway culminates in increased survival and proliferation, similar to the MAPK pathway. The AKT pathway does play a larger role in cell survival compared to MAPK (25).

2.2.3 <u>Receptor tyrosine kinases as targets for therapy</u>

For cancers that are driven by aberrant receptor tyrosine kinase signaling, small molecule inhibitors of the receptors themselves, along with inhibitors of downstream effector molecules, have been developed and demonstrated success in treating certain cancers (Fig 6). Tyrosine kinase inhibitors work by blocking the ATP-binding site of the receptor, acting as a competitive inhibitor. The kinase is then unable to phosphorylate itself or initiate downstream signaling. Major examples include targeting EGFR in breast cancer (HER2), glioblastomas, colorectal cancer, and non-small cell lung cancer; VEGFR in renal cell cancer and GISTs; PDGFR in GISTs, oligodendrocytomas, and squamous cell carcinoma. Blocking the signaling transduction pathways reverses the constant stimulatory input to the cancer cell, along with inhibiting the inhibitory signals on apoptosis, pushing the cancer cells towards cell death. While tyrosine kinase inhibitors and small molecule inhibitors have shown great promise in the treatment of cancer and can have marked initial results, development of resistance is often rapid (26,27).

2.2.4 <u>CD117 (c-KIT) receptor in cancer</u>

The KIT proto-oncogene, or stem cell factor, encodes a tyrosine kinase receptor known to be linked to the classic survival and proliferation signaling pathways, predominantly MAPK and AKT. C-KIT plays an important role in stemness of cells and differentiation. Deregulation of c-KIT that is oncogenic include overexpression of the receptor and gain of function activating mutations, though loss of function mutations have been found in some human cancers including melanoma, thyroid carcinoma, and breast cancer (28). Additionally, gain of function mutations in c-KIT in metastatic melanoma induces apoptosis, while in the majority of cases

activating mutations lead to development of tumors. The most well-known cases in which mutated c-KIT leads to the development of cancer is mastocytosis and gastrointestinal stromal tumors (GISTs), and this is true for both humans and dogs. Mutations leading to mastocytosis in humans primarily involve codon 816 in exon 17 of the c-KIT gene, which encodes a portion of the kinase domain (29). Mutations in GISTs typically involve exon 11 of the juxtamembrane domain. In canine mast cell tumor, internal tandem duplicates, point mutations, and deletions have been found in the juxtamembrane domain, with internal tandem duplication c-KIT mutations being the most common. These mutations are found in exons 11 and 12 in 30-50% of cases (30). Exon 11 c-KIT mutations were found in 73.9% of canine GIST cases in one study (31). It is possible that there are other canine cancers that rely on a driver mutation in the c-KIT gene. In a study evaluating human malignant endocrine pancreatic tumors, 92% of tissue evaluated was positive for c-KIT via IHC (32). While it is not known if this receptor is mutated in this tumor type, it may still represent a targetable molecule (Fig 7).

2.2.5 Platelet derived growth factor receptor in cancer

Platelet derived growth factor plays a major role in angiogenesis and maintenance of stroma. Processes that are driven by activity of these receptors in the adult include stimulation of fibroblasts and smooth muscle cells in wound healing, along with pericyte recruitment and development of vascular smooth muscle in vasculogenesis (33) (Fig 7).

In some malignancies, genetic mutations have been identified that lead to constitutive activation of the receptors. For example, amplification of the PDGFR alpha has been found in glioblastoma, and fusion proteins have been shown in cases of chronic myelomonocytic

leukemia (33). Autocrine PDGF receptor activation has also been shown to contribute to the growth of glioma cells. In such cases, PDGF receptors have been linked to the classic survival and proliferation cell signaling pathways.

In human GISTs, the predominant genetic mutation that leads to the development of GISTs is an activating mutation in the receptor tyrosine kinase c-kit, and tyrosine kinase inhibitors of the KIT receptor have been employed in treating this type of cancer in humans. It has been shown that in about 35% of GISTs that have wild type KIT, there is an activation mutation in PDGFR alpha (34). This activating mutation is homologous to that which is seen in KIT and FLT3 in human mast cell disease, acute myeloblastic leukemia, and seminomas. PDGFR alpha mutant GISTs show activation of AKT, MAPK, and STAT pathways. Other examples include overexpression of PDGF in human colorectal cancer cells, suggesting an autocrine role in cancer development (35). Overexpression of PDGFR has been shown in human non-small cell lung cancer and the use of PDGFR inhibitors induces apoptosis in vitro (36). PDGF and its receptor were found to be highly expressed in a human osteosarcoma cell line, and treatment with a PDGFR inhibitor led to apoptosis of the cells in vitro, and decreased phosphorylation of AKT (37). It is also interesting to note that the retroviral oncogene v-sis is derived from the PDGF B chain gene (38).

The expression of PDGF receptors has been evaluated in human gastrointestinal neuroendocrine tumors via immunohistochemistry. In one study, PDGFR alpha was found to be expressed in the majority of midgut carcinoids and endocrine pancreatic tumors, both by the tumor and the surrounding stroma, while PDGFR beta showed negative staining in all tumor

tissue but was variably positive in the surrounding stroma (39). In a later study, all

neuroendocrine tumors evaluated were positive for PDGFR alpha via IHC and the majority of

stroma was positive. While some of the neuroendocrine tumors in this study stained positive

for PDGFR beta, all four of the insulinomas evaluated were negative while the stroma stained

positive (32).

2.2.6 Vascular endothelial growth factor receptor in cancer

Similar to PDGF, VEGF and its receptors are classically known for stimulating

angiogenesis, and tumor cells have mechanisms to increase release of VEGF even in normoxic

conditions (40) (Fig 7). VEGF is known to be the primary tumor angiogenesis factor, and the role

of VEGF and its receptors in angiogenesis and lymphangiogenesis has dominated the VEGF

research field. In addition to its role in angiogenesis, VEGF has also been implicated in affecting

immune cells in the tumor microenvironment, and regulating fibroblasts in the tumor stroma.

However, VEGFR has also been demonstrated to be present on cancer cells, and expression has

been shown in a multitude of human cancers (41). VEGFR activity has been linked to increased

expression of enhancer of zeste homolog 2 (EZH2), which ultimately leads to downregulation of

tumor suppressor genes (42). Significant overexpression of VEGF has been shown in colorectal

cancer cells and in human non-small cell lung adenocarcinoma cells (35,43). The overexpression

of both VEGF and its receptors was found in pancreatic adenocarcinoma cell lines, suggesting a

possible autocrine mechanism (44).

Intense immunohistochemistry staining for VEGF has been demonstrated in human

gastrointestinal carcinoids and pancreatic endocrine tumors (45). In a study evaluating

expression of VEGF receptors in neuroendocrine tumors, it was found that VEGF receptors were expressed in the majority of tumors, with no significant expression by normal endocrine cells (46). In another IHC study, pancreatic neuroendocrine tumors had the highest expression of VEGFR 1 and 3 in a group of gastrointestinal neuroendocrine tumors (47).

2.2.7 Removing constitutive signal and induction of apoptosis in various cancers

Activating driver mutations of RTKs can drive the development of cancer, and it could be expected that removal of this constitutive signal would cause stasis of the tumor rather than cell death. However, various experiments have demonstrated that blocking the driver signaling pathway can induce apoptosis in cancer cells. Early studies into treatment of canine mast cell tumors with c-KIT inhibitors showed that cell cycle arrest was not increased in treated cells (via propidium iodide (PI) staining and flow cytometry) depending on the type of mutation within the receptor, but that there was increased PARP cleavage in KIT-mutant cell lines following treatment (48). Inhibition of multiple RTKs via targeting their carbohydrate moieties leads to induction of apoptosis in human cervical, colorectal, and lung carcinoma cells, evidenced by Annexin-V and PI staining (49). Treatment of gastric cancer cells with imatinib resulted in induction of apoptosis demonstrated by Annexin-V and PI staining (50). While a multitude of in-vitro studies have shown the ability of TKIs to induce apoptosis in cancer cell lines, many of them do not link the mechanism by which the induction of apoptosis is occurring.

Sunitinib has shown the ability to induce apoptosis in medulloblastoma tumor cells, secondary to the inhibition of the STAT3 and AKT signaling that lies downstream of PDGFR (51). Sunitinib has also been shown to induce apoptosis and increase expression of proapoptotic

proteins in thyroid cancer cells (52). It has been demonstrated that activation of BCL-2

interacting mediator of cell death (BIM) is required for TKIs to induce apoptosis. Imatinib is a

TKI that targets the fusion protein BCR-abl, along with targeting the RTKs PDGFR and c-KIT.

Imatinib kills BCR-Abl positive leukemic cells by activating the pro-apoptotic proteins BIM and

BAD, both via increased transcription and post-translational activation (53). Additionally, the

pro-apoptotic protein BAX displays translocation from the nucleus to the cytoplasm in erlotinib-

treated H3255 lung cancer cells, and multiple studies into the apoptotic mechanism in TKI-

treated EGFR mutant cells suggest that EGFR-TKI induced-apoptosis is via the intrinsic pathway

(54). In colorectal cancer cell lines with a BRAF V600E mutation, it was shown that treatment

with MEK inhibitors led to cell death, and that BIM was de-phosphorylated following treatment.

These cells are dependent on constitutive activation of MAPK signaling, and inhibition of this

signaling leads to increased activity of BIM and a push towards apoptosis (55).

Apoptosis is ultimately mediated by caspases, of which there are 12 and are broken into

initiator and effector caspases. Effector caspases ultimately lead to degradation of the inhibitor

of caspase-activated DNase (ICAD), inducing the activation of endonucleases and proteases

resulting in the degradation of chromosomal DNA and cellular proteins. Caspases can be

activated by an intrinsic or extrinsic pathway. One way in which the intrinsic pathway can be

triggered is by the removal of growth factor signaling, and cancer cells that developed due to

over-active growth factor signaling become dependent on this signaling for survival. Removal of

this signaling cascade via TKIs can induce the intrinsic apoptotic pathway, or at least push a cell

towards this induction. The intrinsic pathway relies on the permeabilization of the

mitochondrial outer membrane, which allows the release of cytochrome C from the

intermembrane space into the cytosol. This step leads to the development of apoptosome, which cleaves and activates caspase 9, eliciting the caspase cascade and inducing apoptosis. The permeabilization of the mitochondrial membrane is tightly regulated by a balance between anti- and pro- apoptotic proteins. The pro-apoptotic proteins are a family called BCL-2. The members of the BCL-2 protein family are regulated both transcriptionally and post-translationally. For example, the BCL-2 protein BAD is phosphorylated by the survival kinase AKT, leading to its inhibition. BIM is phosphorylated by ERK, leading to its degradation, and pushing the cell away from apoptosis. Constant stimulation of these pro-survival pathways inhibits apoptosis, and blocking these pathways with TKIs removes this apoptosis-inhibition (25). (Fig 8).

2.2.8 Sunitinib in human pancreatic endocrine tumors

Sunitinib is a tyrosine kinase inhibitor that acts at the VEGFR 1-3, PDGFR alpha and beta, c-KIT, and FLTK-3 receptors. In a phase 1 study, antitumor activity was seen in patients with renal cell carcinoma, GISTs, and one patient with a neuroendocrine tumor (56). In a phase II study assessing patients with intestinal carcinoids or pancreatic neuroendocrine tumors, the highest responses were seen in patients with pancreatic endocrine tumors (predominantly stable disease, though even those that did not reach criteria for partial response were reported to have some shrinkage) (57). In a phase III study evaluating clinical efficacy and safety of sunitinib in cases of human pancreatic endocrine tumors, the progression-free survival was significantly longer for study patients compared to control patients. The majority of study patients achieved stable disease, however there were two complete responses and six partial responses observed. Of those that were considered stable disease, the majority still saw some

decline in their disease burden. These data suggest that neuroendocrine tumors may be more sensitive to inhibitors of c-KIT, VEGFR, and PDGFR, all of which have been shown to be expressed in these tumor types (58).

2.2.9 Toceranib phosphate (Palladia) in veterinary medicine

Toceranib is an indolinone tyrosine kinase inhibitor first studied in the early 2000's. The earliest indolinone small molecule inhibitor was semaxanib, which targeted singly VEGFR2, due to initial concern that broad targeting of tyrosine kinase receptors might lead to unacceptable toxicities. Clinical experience with subsequent TKIs found these concerns to be unwarranted. Toceranib (SU11654) was designed to inhibit VEGFR, PDGFR, FGFR, and KIT family members. Toceranib may also be able to inhibit RET, based on its molecular similarity to sunitinib. Early in vitro studies of toceranib focused on its function against the c-KIT mutation in canine mast cell neoplasia. The compound was shown to be able to inhibit the growth of a canine mast cell tumor cell line known to harbor an activating c-KIT mutation through cell cycle arrest and apoptosis, at concentrations readily achievable in vivo (59). Since development, toceranib phosphate has shown clinical benefit in a plethora of solid tumors, theoretically due to inhibition of angiogenesis (60). The highest response rates were seen in cases of apocrine gland anal sac adenocarcinoma (AGASACA), thyroid carcinoma, and head and neck carcinomas. While toceranib's activity on angiogenesis may lead to clinical benefit in many tumor types, it is possible that there are dysregulated RTKs that are currently unidentified in certain tumor types, that toceranib is capable of inhibiting. For example, RET and VEGFR2 are both expressed in AGASACA, though it is not known if these are drivers in the etiology of this disease. While toceranib can be highly effective initially, predominantly in cancers in which a known target is a

driver such as mast cell tumors, eventually resistance develops. The underlying mechanisms for this resistance is unknown. Development of resistance to small molecule inhibitors in human medicine is frequent as well, with mechanisms of resistance including development of new geneticc mutations, up-regulation of driver proteins, development of multi-drug cellular efflux, and activation of alternative growth factor pathways (61).

2.2.10 <u>Palladia and canine endocrine tumors, including insulinomas</u>

Additional clinical studies have shown benefit in cases of canine neuroendocrine tumors, including 5 cases of canine pheochromocytomas that resulted in predominantly extended stable disease, 42 dogs with thyroid carcinoma that had a clinical benefit rate of 88.4% (majority partial response or stable disease), and 18 of 27 dogs with heart base tumors that achieved stable disease, with 2 of the 27 achieving partial remission (62,63,64). Of these, the expression of RTKs has only been demonstrated in thyroid carcinoma (65). There is a small number of low-powered clinical studies assessing outcome of cases of canine insulinoma treated with Palladia. One of these studies credits the phase III study assessing sunitinib in cases of human pancreatic neuroendocrine tumors as the motivation for the study (66). In each of these studies, patients received established medical treatments, including prednisone and octreotide, in addition to Palladia (66,67). One study demonstrated an overall response rate of 66.7%, with the majority of patients achieving stable disease, similar to that seen in humans (66). Another study demonstrated a significant difference in median overall survival time between a control group treated with medical management alone versus medical management with the addition of Palladia (67). Though these studies suggest a potential clinical benefit, they do not explore the mechanisms underlying this possible benefit. One case report speculated on

the role of VEGFR in insulin secretion and proliferation of beta cells though this mechanism has not been evaluated in canine insulinoma (68).

2.3 Procaspase-3 and caspase-3

2.3.1 Mechanisms of action

Caspase-3 is a major executioner protein of apoptosis. Sufficient procaspase-3 activation to caspase-3 is often regarded as the point of no return for a cell entering into apoptosis. Active caspase-3 initiates a chain of proteolysis, both by direct proteolysis of key proteins necessary for cell survival, along with proteolysis and activation of other enzymes responsible for cleavage of cellular structures such as DNA (69).

Procaspase-3 is activated into caspase-3 via proteolysis at Asp9, Asp28, and Asp175. This cleavage is typically achieved by caspase-8 or caspase-9, or via auto-cleavage by procaspase-3 or another caspase-3 enzyme. The roles of caspase-3 and procaspase-3 are vital for the fate of the cell, and their activities are regulated by the levels of expression and post-transcriptional modifications of procaspase-3. The procaspase-3 protein is encoded by the CASP3 gene. The expression of this gene is regulated by the E2F family of transcription factors. In the presence of growth factor binding to extra-cellular receptors, signal transduction leads to CDK 4 and 6 kinases which form a complex with cyclin D and cause subsequent phosphorylation of retinoblastoma. Retinoblastoma is complexed to E2F in the absence of growth signals, and once phosphorylated dissociates from E2F. E2F is then free to bind to DNA and to act as a transcription factor, increasing the expression of procaspase-3 (25,69).

Once translated, procaspase-3 exists as a zymogen, albeit with its own intrinsic proteolytic activity. The proteolytic activity of procaspase-3 is, however, about 200-fold less compared to its active form caspase-3. While its intrinsic activity is low, procaspase-3 has the ability to auto-catalyze, which would allow compounding activation within the cell. To prevent this, the activity and activation of procaspase-3 is inhibited by a labile pool of zinc ions that ubiquitously exist within a cell (70).

Caspase-3, once activated, can be inhibited by multiple post-translational modifications via the addition of peptide groups to the catalytic domain or cysteine residues, or via serine phosphorylation. There are also multiple anti-apoptotic proteins that mediate ubiquination of the executioner caspases. The enzyme is also inhibited by the labile pool of zinc similar to its zymogen counterpart (70).

2.3.2 Procaspase-3 in cancer

Interestingly, the over-expression of procaspase-3 in cancer cells has been reproducibly demonstrated across a range of cancer types (70). As mentioned, the expression of procaspase-3 is under the control of the E2F transcription factors. As is a common theme, players of the cell cycle are oden highly dysregulated in cancer. Up-regulation of cyclins and their kinases often is seen, and overexpression of CDK4 and 6 along with cyclin D leads to upregulated activity of E2F, which ultimately leads to overexpression of multiple genes including procaspase-3. The abundance of procaspase-3 molecules produced in this manner can overwhelm the inhibitory zinc pool, leading to an increase in auto-activation of procaspase-3 to caspase-3 molecules, though in a sub-lethal manner as these cancer cells continue to grow and divide despite this. It

is paradoxical that the inactive form of an executioner apoptotic enzyme is overexpressed in cancer cells, however it may simply be a by-product of a dysregulated signaling cascade that overall is sub-compensatory to prevent tumorigenesis. Alternatively, it has been suggested that an increase in sublethal activity of procaspase-3 and caspase-3 can actually be beneficial to the cancer cell. This can be thought of as akin to the carcinogenic potential of traditional anti-cancer therapeutics such as chemotherapy and radiation therapy – these "treatments" cause hundreds if not thousands of genetic mutations which predominantly lead to apoptosis and cell death, however sublethal mutagenesis would have a high probability of leading to cancerous cells. DNA repair machinery is preferentially cleaved and rendered inactive by the proteolytic activity of caspase-3. If a cell does not then commit to apoptosis, genomic instability is likely to occur as a result of the incapacitated DNA repair proteins, in addition to the increased activity of endonucleases directly activated by caspase-3. Genomic instability via caspase-3 activity can be advantageous to tumorigenesis, and this has been demonstrated in experiments in which DNA damage was created in the context of absent or incompetent caspase-3, leading to decreased genomic instability and inhibited tumorigenesis compared to controls (71). Other reports have shown extensive DNA damage as a result of sublethal treatment with inhibitors of apoptosis-inhibitors, that was prevented with pan-caspase inhibitors, along with the induction of extensive DNA damage with sublethal pro-apoptotic agents (72). Therefore, there is ample evidence that caspase-3 promotes DNA damage and oncogenic transformation of a cell if there is not a sufficient amount to commit the cell all the way through apoptosis.

Traditional cytotoxic and many precision medicine strategies rely and converge on induction and completion of apoptosis. These strategies most often function at upstream points

in apoptotic pathways. Cancer cells often develop resistance to these strategies and evade

apoptotic signaling at various points within the apoptotic pathways. In many cancer types,

there is a vulnerable pool of procaspase-3 potentially lending itself to therapeutic exploitation

that is located at a more central point of apoptosis.

2.3.3 Procaspase-activating compound 1 (PAC-1)

PAC-1, or procaspase activating compound 1, was discovered in Paul Hergenrother's lab

during a process that screened chemicals for anti-cancer potential. This molecule, when

delivered to cancer cells, signals the cells to self-destruct by activating the executioner protein,

procaspase-3 into caspase-3. This molecule has been shown to induce apoptotic cell death in

vitro (73). Both caspase-3 and procaspase-3 are inhibited by zinc ions, and it has been

determined that PAC-1 binds to zinc in a 1:1 ratio, thereby sequestering the inhibitory ions and

allowing for more activation of caspase enzymes. PAC-1 is additionally able to enhance the

activity of procaspase-7 and caspase-7 through the same release of zinc inhibition (Fig 9). PAC-1

is able to bind and sequester zinc ions through its ortho-hydroxy N-acylhydrazone motif, and

alteration of this motif abolishes the ability of the compound to chelate labile zinc. PAC-1 has

shown to not be cytotoxic to non-cancerous cells, likely due to a decreased pool of procaspase-

3 in these cell populations, and studies of its use in canines with various malignancies have

shown an appropriate safety profile (74,75,76).

PAC-1 has shown anti-cancer effects in vitro as a sole agent, and a recent phase 1 study

demonstrated prolonged stable disease in a multitude of solid tumors, with the most

provocative results in five cases of neuroendocrine tumors including 64.3% reduction in a

patient with a pancreatic NET and 36% reduction in an ileal NET (77).

2.3.4 PAC-1 synergism

PAC-1 has been studied in vitro and in animal models in conjunction with traditional

therapies, where significant synergism with cytotoxic therapies has been demonstrated (75, 76,

78). These studies have included both in vitro and in vivo models of synergism with traditional

cytotoxic chemotherapeutics and ionizing radiation therapy.

Small molecule inhibitors that target aberrant driver kinases can lead to dramatic anti-

cancer effects, though unfortunately these effects are most often short-lived due to multiple

potential mechanisms that re-activate downstream survival and proliferation signaling

pathways. Combinatorial therapy with additional inhibitors that target various players in these

signaling pathways can delay the onset of resistance, but not abolish it. Resistance often is

associated with re-activation of the MAPK pathway. The BRAF inhibitor vemurafenib has been

shown to induce apoptosis via increased activity of caspase-3, and the addition of PAC-1 to

V600E BRAF melanoma cell lines treated with vemurafenib led to synergistic cell killing,

specifically via apoptosis (79). The percent of V600E BRAF cells entering apoptosis after

treatment with either vemurafenib or PAC-1 ranged from 0-10%, however combination

treatment achieved percentages between 40-50%, which is much more than would be

expected with simple additive effects. It was demonstrated that this synergistic activity was

mediated through significant increase in caspase-3 activity. A follow up study demonstrated in

vitro that PAC-1 is able to enhance caspase-3 activity of kinase inhibitors that target driver

mutations in EGFR, EML4-ALK, and BCR-abl (80). Previous studies have shown that MEK1 and

MEK2 are cleaved and destroyed by caspase-3, and that MEK 1 and 2 are the only kinases that

phosphorylate ERK 1 and 2 (81,69). When small molecule inhibitors block the MAPK pathway

leading to decreased phosphorylated ERK 1 and 2, there is decreased negative feedback on the

pathway, leading to hyperphosphorylation and rebound activity of MEK (82,83). It has been

demonstrated that degradation of MEK 1 and 2 secondary to PAC-1 treatment abolishes this

rebound effect, therefore leading to synergistic apoptotic cell death (80).

Chapter 3

Materials and Methods

3.1 Cell Lines

A canine insulinoma cell line was provided by Floryne Buishand and used in this study. A human BON cell line was used for comparison studies. C2, Jones, and Park cell lines were used for positive controls. Cells were cultured at 37 degrees C in Roswell Park Memorial Institute (RPMI) medium supplemented with penicillin (100 IU/mL), streptomycin (100 IU/mL), and 10% fetal bovine serum (FBS), and 200 mcg of growth hormone in a humidified atmosphere supplemented with 5% CO_2. Cell cultures were maintained in subconfluent monolayers and passaged every two to three days as necessary.

3.2 Reagents and Antibodies

Canine insulinoma tissue slides were obtained from the University of Illinois Veterinary Diagnostic Lab. A canine insulinoma tissue microarray was obtained from Floryne Buisand. Anti-PDGFR beta (rabbit monoclonal) was purchased from Cell Signaling. Anti- procaspase 3 (rabbit monoclonal) and anti-VEGFR2 (rabbit polyclonal) were purchased from Abcam. Anti-CD117 (rabbit polyclonal) antibody was purchased from Agilent Technologies. Anti-Erk1/2 (rabbit monoclonal), anti-phospho Erk1/2 (Thr202/Tyr204) (rabbit monoclonal), anti-AKT (rabbit polyclonal), anti-phospho AKT (Ser 473) (rabbit polyclonal), and anti-PARP (rabbit polyclonal) antibodies were purchased from Cell Signaling. Anti-beta actin antibody (mouse monoclonal) was purchased from Abcam. Horseradish peroxidase conjugated anti-mouse and anti-rabbit were purchased from GE Healthcare, UK.

Toceranib phosphate was obtained from MedChemExpress and kept at stock concentration of 10 micromolar. Procaspase activating compound 1 (PAC-1) was obtained from the laboratory of Paul Hergenrother.

3.3 Immunohistochemistry for expression of treatment targets

Nineteen primary canine insulinoma and sixteen metastatic insulinoma tissues (seven of which were matched) were originally collected during routine clinical care, and retrieved from the University of Illinois's Veterinary Diagnostic Laboratory for immunohistochemical assessment. Slides were treated with either Borg Decloaker (PDGFR) or Diva Decloaker (Procaspase-3, c-KIT, VEGFR2). Slides were then blocked with Peroxidazed 1 for five minutes. A secondary block with Background Punisher was performed for ten minutes. Slides were then incubated with primary antibody: PDGFR (1:00, 12 hours), procaspase-3 (1:300, 30 minutes), c-KIT (1:100, 30 minutes), VEGFR2 (1:100, 14 hours). Slides were then incubated with a biotinylated secondary antibody for 30 minutes, then with the chromogen IP FLX DAB for five minutes. Slides were counterstained with hematoxylin (Cat hematoxylin). All stains and reagents used were obtained from Biocare Medical (Pacheco, CA). All samples were evaluated by a single individual (ALS) and relative staining intensity was numerically scored ("0" for no staining, "1" for weak staining; "2" for moderate staining; "3" for strong staining). In addition to naturally occurring tumor samples, procaspase-3 staining was evaluated in a canine insulinoma tissue microarray.

Canine lymph node tissue was used as positive control for antibody against procaspase-3, canine mast cell tumor tissue was used as positive control for antibody against c-KIT, canine

testis tissue was used as positive control for antibody against PDGFR beta, and canine

mammary carcinoma tissue was used as positive control for antibody against VEGFR2.

3.4 Cell Protein Collection

Cells were grown in culture until 80% confluence was attained. Media was removed and

cells were washed once with PBS. Cells were exposed to trypsin for five minutes until detached

from culture plate. Media was added to neutralize the trypsin. The mixture was centrifuged at

1,500 g at room temperature for five minutes. Supernatant was removed and the cell pellet

homogenized in one mL PBS and transferred to a 1.5 mL Eppendorf tube before centrifuging at

10,000 g at room temperature for five minutes. Supernatant was removed and discarded. Cell

pellets were stored at -80 degrees C until protein collection. Cell pellets were homogenized

with Mammalian Protein Extraction Reagent (M-PER, Pierce, Rockford, IL) and mixed with

Pierce protease inhibitor cocktail solution (or protease and phosphatase inhibitor cocktail for

phosphorylation experiments) (diluted 1:100 for final working solution). Homegenates were

placed on ice for thirty minutes followed by sonication three times for ten seconds each. The

samples were centrifuged at 10,000 g for 10 minutes. The supernatant was collected and stored

at -20 degrees C until quantification (with the exception of protein used for phosphorylation

experiments which were quantified and used immediately following collection). Cellular protein

concentrations were determined using a standard assay kit (Bicinchoninic Acid Protein Assay,

Pierce, Rockford, IL).

3.5 <u>Western Blot Analysis</u>

3.5.1 <u>Evaluation of phosphorylation status of Erk and AKT in treated canine insulinoma cells</u>

The canINS cell line was grown to 85% confluence. Cells were then exposed for 24 hours to experimental conditions including: 1) stock media alone, 2) 2.5 uM PAC-1, 3) 10 uM of PAC-1, 4) 0.25 uM toceranib phosphate, 5) 2.5 uM PAC-1 combined with 0.25 uM toceranib phosphate, 6) 10 uM PAC-1 combined with 0.25 uM toceranib phosphate. Media was removed and cells were washed once with PBS followed by exposure to trypsin for five minutes. Cells were collected for protein collection. For protein expression analysis, 20 ug samples were electrophoresed on 10% polyacrylamide gel and then electrophoretically transferred to nitrocellulose membrane. The membranes were blocked with 1X tris-buffered saline, 0.1% tween (TBST) with 5% milk for one hour at room temperature. Western blot analysis was performed using Erk, phosphor-Erk, AKT, and phospho-AKT antibodies at a concentration of 1:1000 (ERK, AKT, p-AKT) and 1:2000 (p-ERK) in TBST with 5% milk. The membrane was washed five times with TBST and probed with the secondary anti-rabbit antibody diluted 1:2000 in TBST with 5% milk. The blots were developed using ChemiDoc XRS+ molecular imaging system (Bio-Rad).

3.5.2 <u>Evaluation of PARP Cleavage in treated canine insulinoma cells</u>

The cells were plated and treated under identical conditions to blots used for evaluation of phosphorylation of Erk and AKT. Western blot analysis was performed using anti-PARP antibody at a concentration of 1:1000 in TBST with 5% milk. The membrane was washed five times in TBST and probed with the secondary anti-rabbit antibody diluted 1:2000 in TBST with 5% milk. The blots were developed using ChemiDoc XRS+ molecular imaging system (Bio-Rad).

3.6 <u>Cell Survival Assay</u>

Canine insulinoma cells were seeded overnight in 96-well plates at a concentration of 1500 cells per 200 uL of media. Upon adherence of cells, 200 uL of additional media containing different experimental conditions was added including stock media with DMSO. Toceranib phosphate was used at concentrations of 0.25 uM, 0.5 uM, 0.75 uM, 1 uM, and 1.25 uM. PAC-1 was used at concentrations of 1.25 uM, 2.5 uM, 5 uM, 7.5 uM, and 10 uM. A third plate was used to assess combination effects.

3.7 <u>SRB Assay</u>

Following treatments at various time points, cell mass was assessed via optical density. 100 uL of cold 10% trichloroacetic acid (TCA) was added to each well and incubated at 4 degrees C for 1 hour. 96 well plates were washed four times with tap water and allowed to dry on benchtop. 100 uL of 0.057% SRB in 1% acetic acid was added to each well and incubated at room temperature for 30 minutes. Plates were washed four times with 1% acetic acid and allowed to dry on benchtop. 200 uL of 10 mM Tris base at a pH of 10.5 was added to each well and incubated at room temperature for 30 minutes. Optical density was then measured at a wavelength of 562 nm.

3.8 <u>Cell Treatment for RNA Sequencing</u>

Three 6-well plates were seeded with canine insulinoma cells at a seeding density of 150,000 cells per well. Following attachment of cells, treatments were added in triplicate. Treatments included DMSO vehicle treatment, Palladia at 0.25 uM, PAC-1 at 2.5 uM and 10 uM,

and combination treatments. Cells were exposed to treatments for six hours prior to RNA collection.

3.9 RNA Collection

Following six hour treatment, media was removed and cells were washed with PBS. Cells were exposed to 700 uL of Trizol for approximately five minutes until cells detached from culture plate. RNA collection kit Direct-zol RNA Miniprep (Zymo Research) used. The liquid was collected and transferred into Eppendorf tubes. 700 uL of 95% ethanol was then added to each sample. Mixtures were transferred into Zymo-Spin columns within collection tubes. Samples were centrifuged at 10,000 g for 30 seconds. Columns were transferred to new collection tubes and the flow-through was discarded. 400 uL of RNA Wash Buffer was added to each sample. 5 uL of DNase I and 75 uL of DNA Digestion Buffer were also added to each sample. Samples were incubated at room temperature for 15 minutes. Follow incubation, 400 uL Direct-zol RNA PreWash added to each column. Samples were then centrifuged at 10,000 g for 30 seconds. The flow-through was discarded and this step was repeated. 700 uL of RNA Wash Buffer was added to each column and samples were centrigued for 60 seconds at 10,000 g. Columns were then transferred to RNase-free tubes. 50 uL of DNase/RNase-free Water was added to each column. Samples were allowed to sit at room temperature for two minutes before centrifugation at 10,000 g for 60 seconds. Samples were frozen at -80 degrees C until use.

3.10 <u>RNA Sequencing</u>

Quality of the sequencing was checked using FASTQC1 (v0.11.8); average per-based read quality scores were over 30 to the end of the 150 bp reads and adapter sequences had already been trimmed, so no additional trimming was needed.

Sequenced reads were pseudo-aligned to NCBI's Canis lupus familiarias Annotation Release 106 transcripts using Salmon2 (v1.5.2) and the ROS_Cfam_1.0 genome as the decoy sequence and with the following options: --seqBias --gcBias --numBootstraps=30 -- validateMappings --recoverOrphans. The transcript-levels counts were then read into R3 (v.4.2.3) and gene-level counts were calculated using the "lengthScaledTPM" method from the tximport package (v1.26.1); this method provides more accurate gene-level counts estimates and keeps multi-mapped reads in the analysis compared to traditional alignment-based method4. Genes that did not have at least 1 count per million mapped reads (CPM) in at least 3 samples were filtered out. Additional normalization to correct for RNA composition was done using the TMM method5 in the edgeR6 package (v3.40.2). The RUVSeq package7 (v1.32.0) was used to estimate 2 additional correction factors for spurious technical noise to help improve biological insights8. Differential gene expression testing was done using the limma-trend method9 from the limma package (v3.54.2) and a 2way ANOVA model with main effects of each drug and interactions between them. Eleven post-hoc pairwise comparisons were made between specific treatment groups and multiple test correction was done globally across all 11 comparisons using the False Discovery Rate10 method.

3.11 <u>Statistical Analysis</u>

All data was evaluated for normality using Shapiro-Wilk test. For cell death induction from Palladia, PAC-1, or combination, 1-way ANOVA was used to evaluate for differences in SRB optical density measurements with the use of Bonferroni's Multiple Comparison post-hoc test. Differences in categorical immunohistochemical staining intensities for targets of palladia (c-kit, VEGFR, and PDGFR beta) or PAC-1 (PC-3) between primary tumors and paired metastatic lesions, a student T test was used. For outcome linked tissue microarray, the influence of PC-3 immunohistochemical staining intensity with overall survival time was analyzed using Kaplan-Meier survival analysis, log rank test. Statistical calculations were performed using commercial software programs (Prism-GraphPad), and $p < 0.05$ was considered statistically significant for all analyses.

Chapter 4

Results

4.1 Target Expression

4.1.1 Expression of targets by canine insulinoma tissue

The expression of the targets of toceranib phosphate and procaspase activating

compound 1 was established in canine insulinoma tissue at a protein level via

immunohistochemistry using antibodies against VEGFR2, procaspase-3, c-KIT, and PDGFR beta

(Fig 10). All expression was seen predominantly in tumor tissue compared to surrounding

normal pancreas. PDGFR beta expression was the weakest of the targets with many tissue

samples staining negative, followed by moderate expression of c-KIT. Tumor tissues exhibited

strong expression of procaspase-3 and VEGFR2. Expression was scored numerically from 0-3

based on intensity of staining. All of the tissues had negative staining or a score of 1 for PDGFR

beta. Of all the samples, 80% scored 1 or 2 for c-KIT staining, 70% of samples scored 2 or 3 for

PC-3 staining, and 50% of tissue scored 3 for VEGFR2 staining.

4.1.2 Expression of targets by canine insulinoma tissue compared to metastatic lesions

Intensity scoring was evaluated across both primary and metastatic lesions to determine

a comparison between expression of targets between these tissue types (Fig 11). Procaspase-3

(p = 0.003), C-kit (p = 0.01), and VEGFR2 (p = 0.01) are significantly expressed in higher amounts

in metastatic lesions compared to the primary tumors. This held true when evaluating matched

primary tumor and metastatic lesions as well.

4.2 <u>Prognostic significance of procaspase-3 expression</u>

An outcome-linked canine insulinoma tissue microarray was evaluated for protein expression of procaspase-3 via immunohistochemistry (Fig 11). Procaspase-3 expression negatively correlated with outcome. Disease free interval (DFI) was significantly shorter in cases that exhibited high expression of procaspase-3 (low/intermediate expressors had a median DFI of 462 days, while high expressors had a median DFI of 44 days, $p = 0.03$).

4.3 <u>Cell survival with toceranib phosphate and PAC-1 treatments</u>

The canine insulinoma cell line was found to be resistant to toceranib as a sole treatment (Fig 12). All concentrations of toceranib used resulted in no significant changes to optical density in SRB experiments, even after 72 hour treatments. Conversely, PAC-1 demonstrated dose-dependent cell death in the canine insulinoma cell line, with 50% cell death achieved around 5 uM for 72 hour treatment. While no significant cell death was achieved with toceranib treatment, PAC-1 cell killing was enhanced when combined with toceranib. This synergism was only identifiable at lower concentrations of PAC-1, as higher concentrations of PAC-1 were so effective at cell killing. A significant decrease in optical density was found between treatment with PAC-1 at 1.25 uM for 72 hours compared to treatment with PAC-1 at 1.25 uM and Palladia at 1 uM for 72 hours.

4.4 <u>Transcriptional Response</u>

There were 27 differentially expressed genes (DEG) that were identified via the RNA sequencing experiments for combination of toceranib at 0.25 uM and PAC-1 at 2.5 uM (Fig 13).

The majority of these genes were found to be upregulated by both treatments. Eight of the 27

DEGs showed expressional changes with combination treatment that do not appear to be

additive. Compared to vehicle treated cells, the genes FILPIL and ADA were upregulated in

toceranib treated cells. FILPIL was also upregulated in PAC-1 treated cells, but ADA expression

was static to downregulated in PAC-1 treated cells compared to control. These genes showed

marked upregulation with combination treatment compared to individual treatment and

vehicle. The genes ANLRD17, CAD, TXNRD1, CPT1A, MDN1, and SRSF2 were mildly

downregulated in the canine insulinoma cell line with exposure to toceranib, and the majority

of these genes were upregulated or static when exposed to PAC-1. The combination treatment

resulted in marked downregulation of these six genes.

4.5 <u>Effects on MAPK and AKT Effectors</u>

Western blot analysis was used to evaluate changes in phosphorylation status of

members of the MAPK and AKT pathways (Fig 14 & 15). ERK was found to be expressed in all

samples, and western blots demonstrated a double band that is consistent with results of this

antibody. Phosphorylated ERK was found to be present at a much higher level in cells treated

with 10 uM of PAC-1 for 24 hours compared to vehicle treated and PAC-1 at 2.5 uM. Toceranib

as a sole treatment resulted in decreased phosphorylated ERK, and the increase in

phosphorylated ERK with high concentration of PAC-1 was abolished in the presence of

toceranib. Similarly, AKT was found to be expressed at similar levels across all treatments.

Phosphorylated AKT was found to be present at the highest amount in cells treated with 10 uM

PAC-1. Toceranib treated cells demonstrated decreased amounts of phosphorylated AKT

compared to vehicle treated and cells treated with PAC-1, and the increase in phosphorylation of AKT was abolished when PAC-1 at 10 uM was combined with toceranib at 0.25 uM.

4.6 <u>PARP Cleavage</u>

Western blot analysis was used to evaluate changes in the amount of cleaved PARP in canine insulinoma cell lines after exposure to treatments (Fig 16). Cleaved PARP was found to be present in higher amounts in PAC-1 treated cells compared to vehicle treated cells. Toceranib treated cells demonstrated minimal PARP cleavage, and combination with PAC-1 demonstrated increased amounts of cleaved PARP present. The cleaved PARP band was the brightest in the cells treated with the combination of toceranib at 0.25 uM and PAC-1 at 10 uM.

Chapter 5

Discussion and Conclusions

Canine insulinomas are neuroendocrine tumors of the pancreas that still require more

successful treatment options for prolongation of good quality life and durable glycemic control.

Tyrosine kinase receptors are being used with increasing frequency in both human and

veterinary medicine. This was the first study to demonstrate the expression of membrane

receptors in canine insulinomas targeted by the tyrosine kinase inhibitor toceranib phosphate,

suggesting its utility in this tumor type. The etiology of canine insulinoma remains unknown,

therefore it is not determined if targeting these specific receptors on the tumor cells can

actually provide any clinical benefit. For cancers in which a known driver mutation in a receptor

tyrosine kinase pathway is present, blockade of the aberrant signal can lead to inhibition of

further propagation and/or cell death. Classic examples of this include targeting mutated c-KIT

in canine mast cell tumors and gastrointestinal stromal tumors. Toceranib phosphate has

shown significant efficacy in treating these types of cancers as it is well established that

toceranib can inhibit the constitutive signal created by these mutated receptors. The other

targets of toceranib, predominantly platelet derived growth factor receptor and vascular

endothelial growth factor receptor, have been implicated in some tumor types. Despite this,

demonstration of driver mutations or mechanism of tumorigenesis is lacking in many tumor

types, including canine insulinoma. That being said, it has been demonstrated that the targets

of toceranib send intracellular signals via proliferation and survival pathways, therefore it can

be hypothesized that even in the absence of targeting a driver mutation, blockade of at least

some input into these signaling pathways can tip the balance of a cell away from proliferation

and survival. It would be expected, then, that blockade of these receptors would likely not be sufficient alone to halt cancer progression or be cytotoxic. Indeed, in our study, it was found that while we were able to demonstrate the presence of toceranib's targets on canine insulinoma cells via immunohistochemistry of canine insulinoma tissue and on a canine insulinoma cell line, treatment of this canine insulinoma cell line with increasing concentrations of toceranib did not lead to meaningful cell death. Previous retrospective studies evaluating the clinical use of toceranib in cases of canine insulinoma have suggested benefit. Additionally, sunitinib has demonstrated clinical benefit in cases of human pancreatic neuroendocrine tumors. In the cases of canine insulinoma however, there were other treatments that were used in conjunction with toceranib and it is difficult to discern what the true clinical benefit was. Additionally, the targets of toceranib include VEGFR and PDGFR which play major roles in angiogenesis. Targeting angiogenesis has been shown to be efficacious in treating cancers, and it is possible that the anti-angiogenic role of toceranib was to be credited for any clinical benefit that was seen in these retrospective canine studies. This may also be the case for sunitinib in human pancreatic neuroendocrine tumors as the majority of responders achieved stable disease, however there were two cases of complete response, six partial responses, and many of the stable disease patients had some decline in their disease burden. Human pancreatic neuroendocrine tumors are known to express targets of sunitinib.

While toceranib alone did not achieve cell death in our cell survival assays, the canine insulinoma cell line was quite sensitive to PAC-1 treatment. This cell line demonstrated dose-dependent cell death at various timed treatments. Interestingly, when low concentrations of PAC-1 were combined with higher dose toceranib, there was a repeatable significant increase in

cell death. It is likely that this combined response is lost at higher doses of PAC-1 because the PAC-1 alone is too effective to notice a difference with the addition of toceranib. PAC-1 is known to function very downstream and central within the apoptotic pathway. It can be hypothesized that blockade of survival input via treatment with toceranib can predispose the cells to being more sensitive to the actions of PAC-1. Given that toceranib has no meaningful effect on cell survival as a sole treatment, the increase in cell death with the combination treatment must be synergistic rather than additive. It is possible that changes in cell signaling secondary to toceranib treatment interacts with the apoptotic pathway in some way, leading to greater cell killing by PAC-1 in this cell type.

The expression of the targets of toceranib were scored both in primary canine insulinomas and metastatic lesions. While PDGFR beta did not show robust staining in any of the lesions, c- KIT demonstrated moderate staining and VEGFR2 demonstrated robust staining in the majority of samples. Interestingly, the metastatic lesions stained more intensely compared to primaries for all stains other than PDGFR beta (as staining for PDGFR beta was largely negative or weak for all samples). This held true for primary tumors and metastatic lesions as separate groups. Additionally, the majority of cases of matched primary tumors and metastatic lesions had staining for c-KIT and VEGFR that were similar or more intense. This suggests that for metastatic lesions, which are more often not surgically amenable, toceranib may provide greater benefit.

Procaspase-3 (PC-3), the target for PAC-1, was also demonstrated to be present in these tumor types via immunohistochemistry. PC-3 showed moderate staining in primary tumors,

however a marked increase in staining was seen in metastatic lesions compared to primary lesions. To add to this, PC-3 was found to be expressed higher in cases with known poorer outcomes via immunohistochemistry on the outcome-linked tissue microarray. Therefore, similar to previous research on the prognostic significance of PC-3 expression in human medicine, it appears that PC-3 expression is negatively correlated with outcome in cases of canine insulinoma. Similar to what was demonstrated with toceranib target staining, the cases that would be expected to have the poorer outcomes (such as the metastatic cases) may derive the most benefit from treatment with PAC-1.

Given that synergistic cell killing was seen with toceranib and PAC-1 treatments, we wanted to investigate potential changes in cell survival and proliferation signaling in cells treated with toceranib and PAC-1 to better elucidate underlying mechanisms of these findings. With toceranib treatment alone, there was a decrease in the amount of phosphorylated Erk and AKT compared to vehicle-treated control cells. This suggests that blockade of the targets of toceranib did provide decreased proliferation and survival signaling within the canine insulinoma cell line, as would be anticipated. Interestingly, PAC-1 at low treatment had no effect on phosphorylation of Erk or AKT, but high concentration of PAC-1 treatment led to increased phosphorylation of both Erk and AKT. While PAC-1 is known to act downstream of these effectors and central to the apoptotic pathway, somehow treatment led to increased upstream signaling in the MAPK and AKT pathways. The increased activation of caspases secondary to PAC-1 treatment leads to cleavage of many cellular proteins, and It is possible that there is some sort of feedback mechanism that is being stimulated or inhibited by the increased activity of effector caspases. Interestingly, this increase in phosphorylation of AKT and Erk via

PAC-1 treatment is abrogated with the addition of toceranib. There was no change seen in phosphorylation of AKT and Erk compared to toceranib treatment alone when PAC-1 was added both at low and high concentrations. Somehow, toceranib is inhibiting whatever signaling mechanism is occurring with PAC-1 treatment that leads to increased activity of AKT and Erk. This is consistent with the theory that toceranib and PAC-1 are both affecting proliferation and survival pathways, leading to synergistic cell killing. The mechanism of synergistic killing must not be at the level of activity of Erk or AKT however, given that phosphorylation of both are the same with combination treatment compared to toceranib treatment alone, and we know that this cell line is resistant to toceranib as a single agent.

Poly (ADP-ribose) polymerase (PARP) is an enzyme that is cleaved by caspase-3 early on in the apoptotic process. The amount of cleaved PARP present following treatments with toceranib, PAC-1, and the combination was evaluated. The amount of cleaved PARP present was found to increase with exposure to PAC-1 compared to vehicle-treated cells. The amount of cleaved PARP was not increased with exposure to toceranib alone, which is consistent with findings that toceranib alone does not lead to apoptosis and cell death. The addition of PAC-1 to toceranib resulted in increased PARP cleavage similar to that which was seen with PAC-1 treatment alone. Therefore, synergistic cell killing must also not be at the level of PARP cleavage and possibly not at the level of caspase activity.

RNA sequencing was performed to assess transcriptional changes in the canine insulinoma cell line after a short exposure to toceranib, PAC-1, or the combination. There were some differentially expressed genes, and some transcriptional changes suggest additive or in

some cases synergistic changes. For example, FILPIL and ADA are genes that were mildly upregulated with exposure to each drug as a single agent, but showed robust upregulation with combination treatment. FILPIL is a gene that has shown to play a role in decreasing the development of metastases and chemoresistance. ADA encodes the enzyme adenosine deaminase, which can deplete adenosine and decrease pancreatic beta cells' proliferative ability. ANKRD17, CAD, and CPT1A are genes that were all downregulated in cells treated with toceranib alone, while they remained mostly static or mildly upregulated in cells exposed to PAC-1 alone. Interestingly, the downregulation of these genes was markedly increased in the combination-treated cells. These effects cannot be additive as PAC-1 led to upregulation in these genes, and the combination led to a much more extreme downregulation compared to toceranib alone. ANKRD17 plays a role in DNA replication and cell cycle progression, so further downregulation would support anti-cancer effects. Similarly, CAD plays a role in biosynthesis of pyrimidine nucleotides, and CPT1A plays a role in mitochondrial energy production. Less DNA building blocks and less energy would also be anti-cancerous.

This study was the first to demonstrate the presence of the targets of both toceranib and PAC-1 in canine insulinoma cells. The expression of these targets was found to be higher in metastatic cases or in cases with poorer outcome. Therefore, pet dogs with a worse prognosis could be those that benefit the greatest from this combination of treatments. PAC-1 and toceranib showed modest synergy at low concentrations, and PAC-1 amplified toceranib-induced transcriptional changes. The safety of both toceranib and PAC-1 have been established in pet dogs, and future studies are needed to assess the safety and efficacy of this combination in pet dogs with insulinoma.
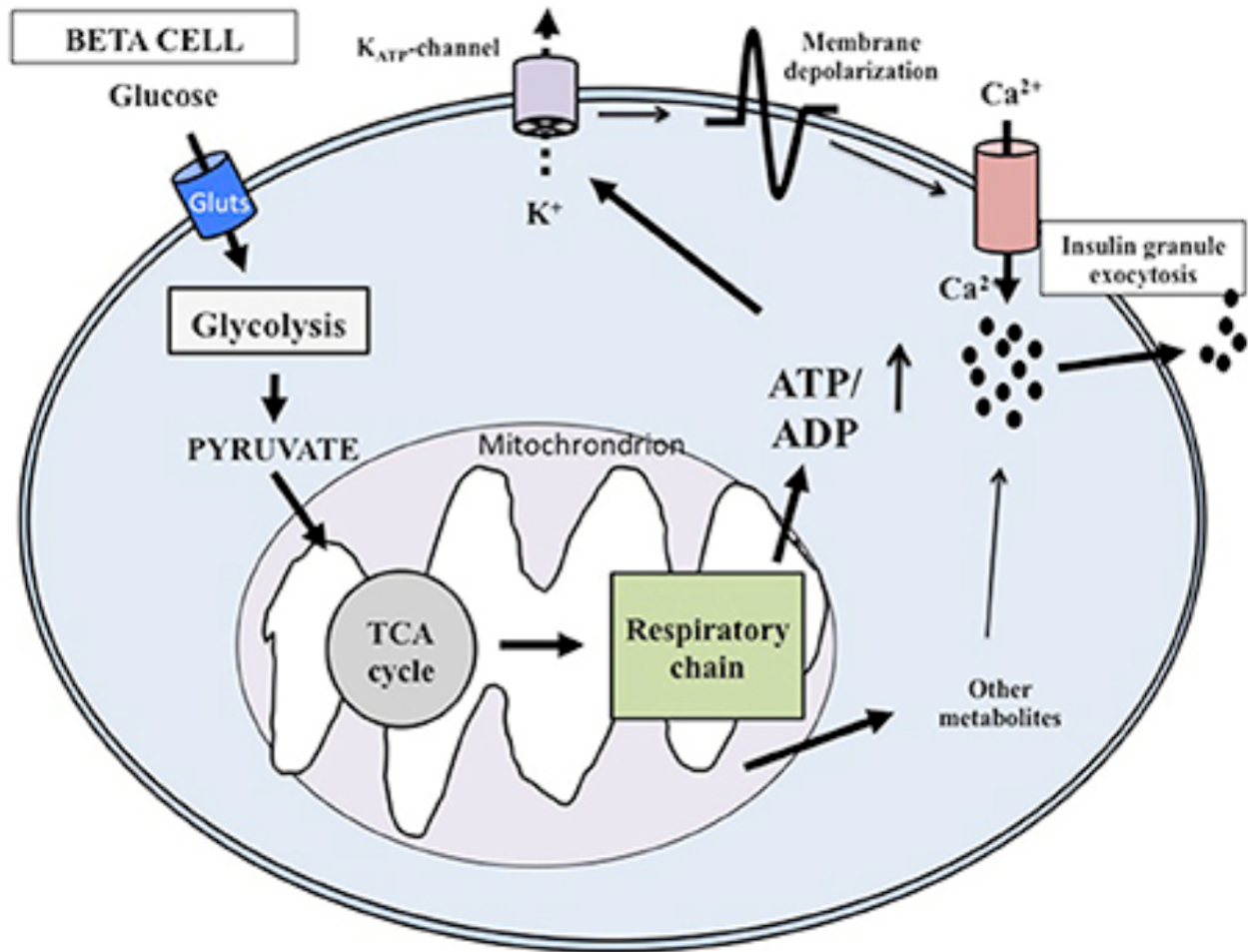
Figures

Figure 1. Glucose handling and insulin release by the pancreatic beta cell – glucose enters the pancreatic beta cells via insulin-independent GLUT2 channels and enters into the TCA cycle, leading to a generation of ATP. This leads to activation of potassium channels and potassium efflux, leading to depolarization of the cell and opening of voltage-gated calcium channels. This intra-cellular calcium stimulates exocytosis of insulin granules and insulin release (Marchew et al, 2017).
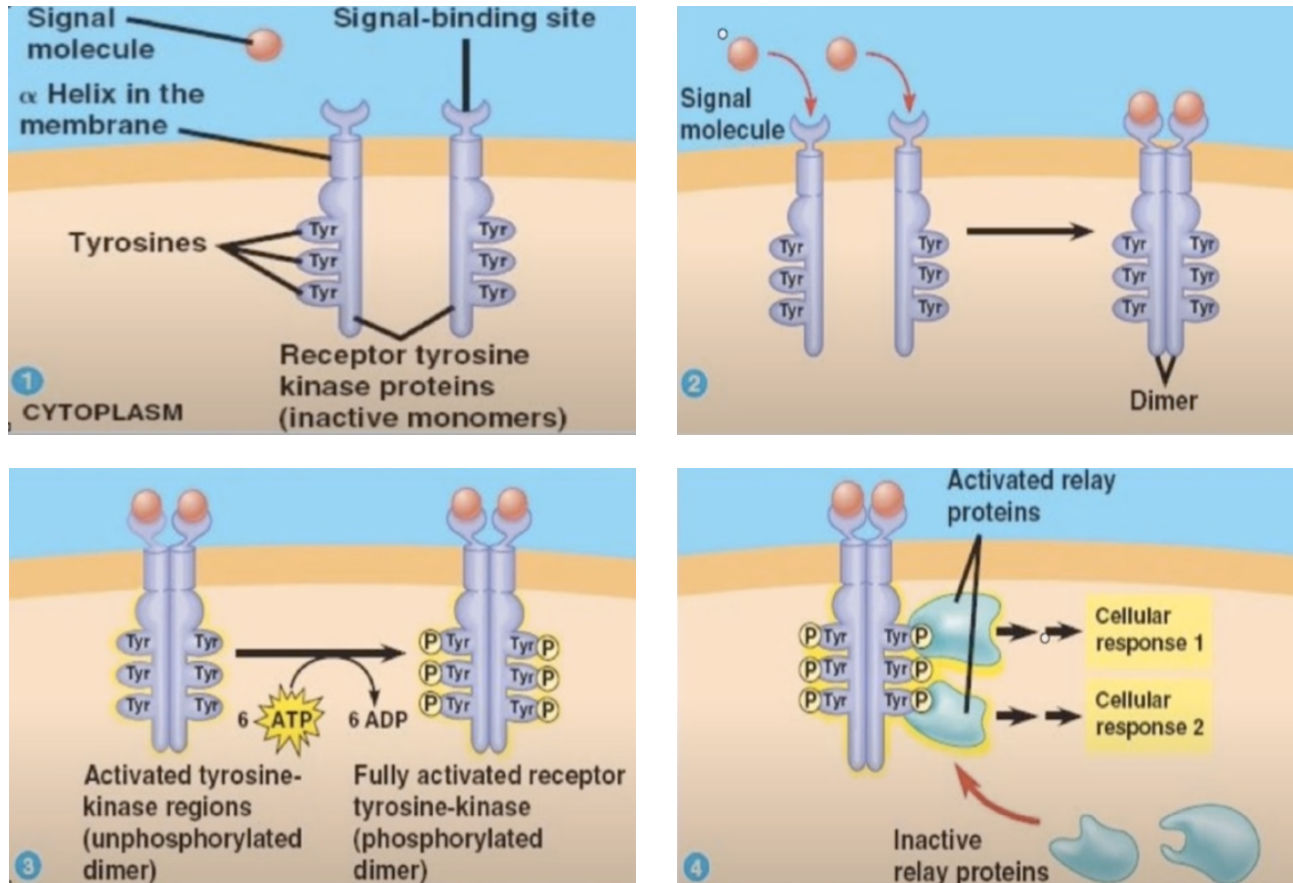
Figure 2. Receptor tyrosine kinases – Receptor tyrosine kinases start out as inactive monomers. Following binding of the ligand site, the monomers come together and dimerize, which leads to phosphorylation of the cytoplasmic tyrosine molecules. Inactive enzymes or relay proteins are brought to the membrane surface and become phosphorylated and activated, which then lead to a cascade of phosphorylation events that terminate in increased expression of factors responsible for growth and proliferation of the cell.
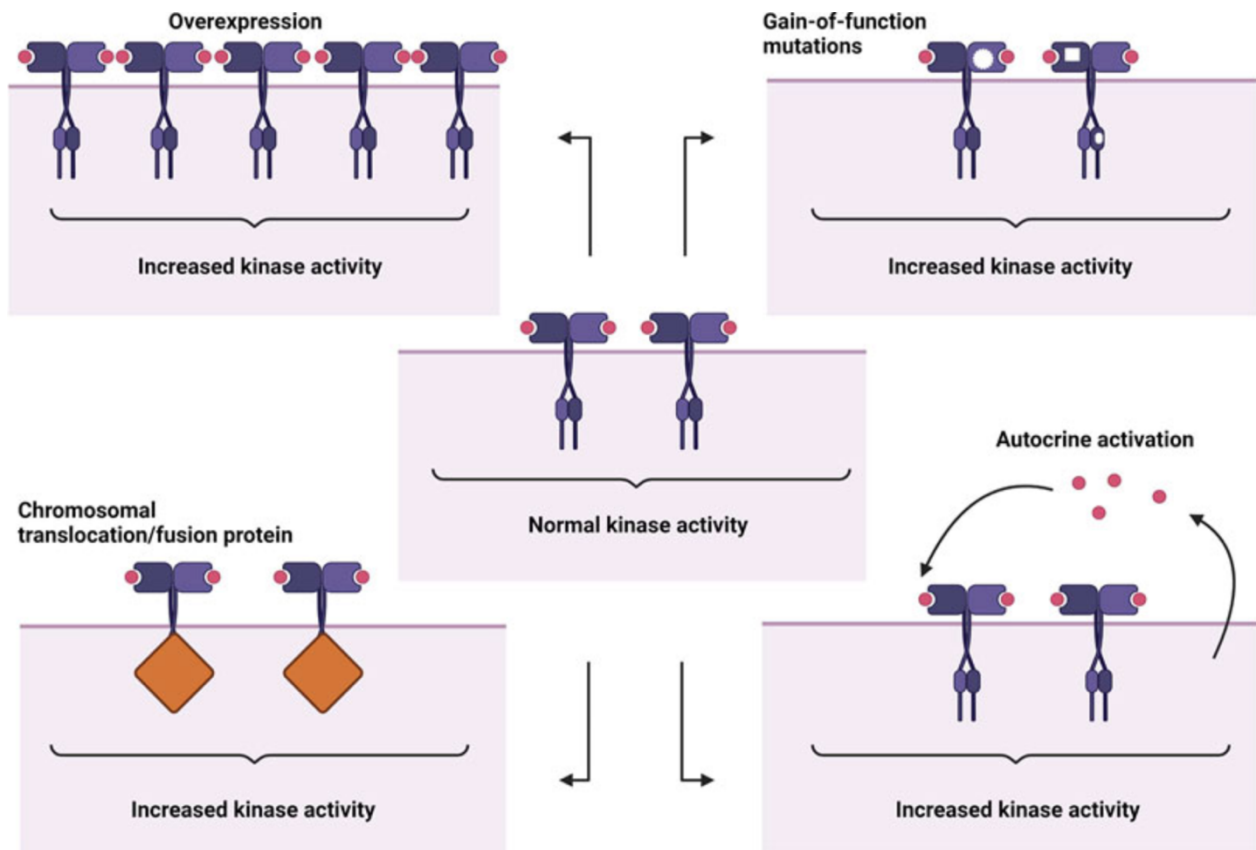
Figure 3. Tyrosine kinase receptors in cancer – Mechanisms by which increased kinase activity can occur leading to the development of hyper-proliferation include overexpression of the receptor, a gain-of-function mutation in the receptor leading to constitutive activation and development of consistent signal, chromosomal translocation or development of a fusion protein that leads to constitutive activation and consistent signal, and autocrine activation of the receptor (Dev et al, 2021).
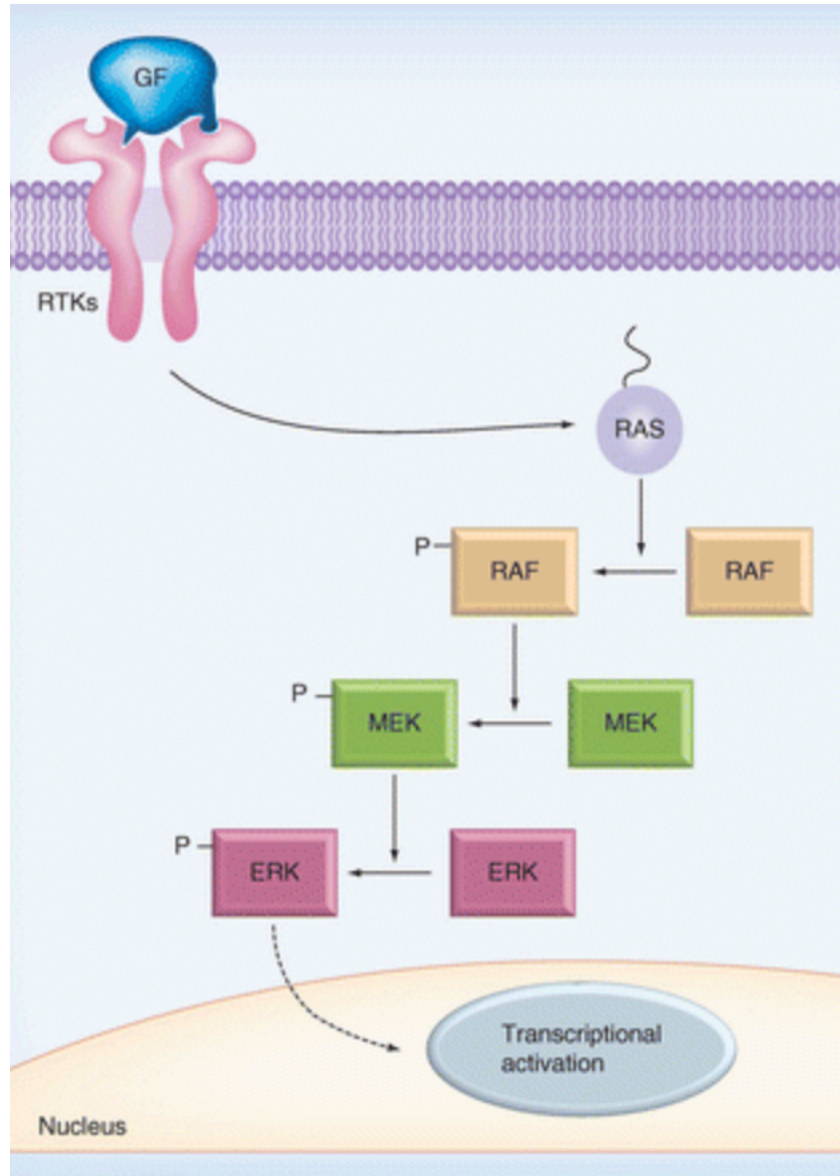
Figure 4. The MAPK Pathway – The MAPK pathway is coupled to many receptor tyrosine kinases. Once activated, there are cytosolic downstream messengers that propagate signal from external to the cell down to the nucleus. Many of the transcriptional changes that occur secondary to activation by Erk involve proliferation and cell survival (Change and Marton, 2014).
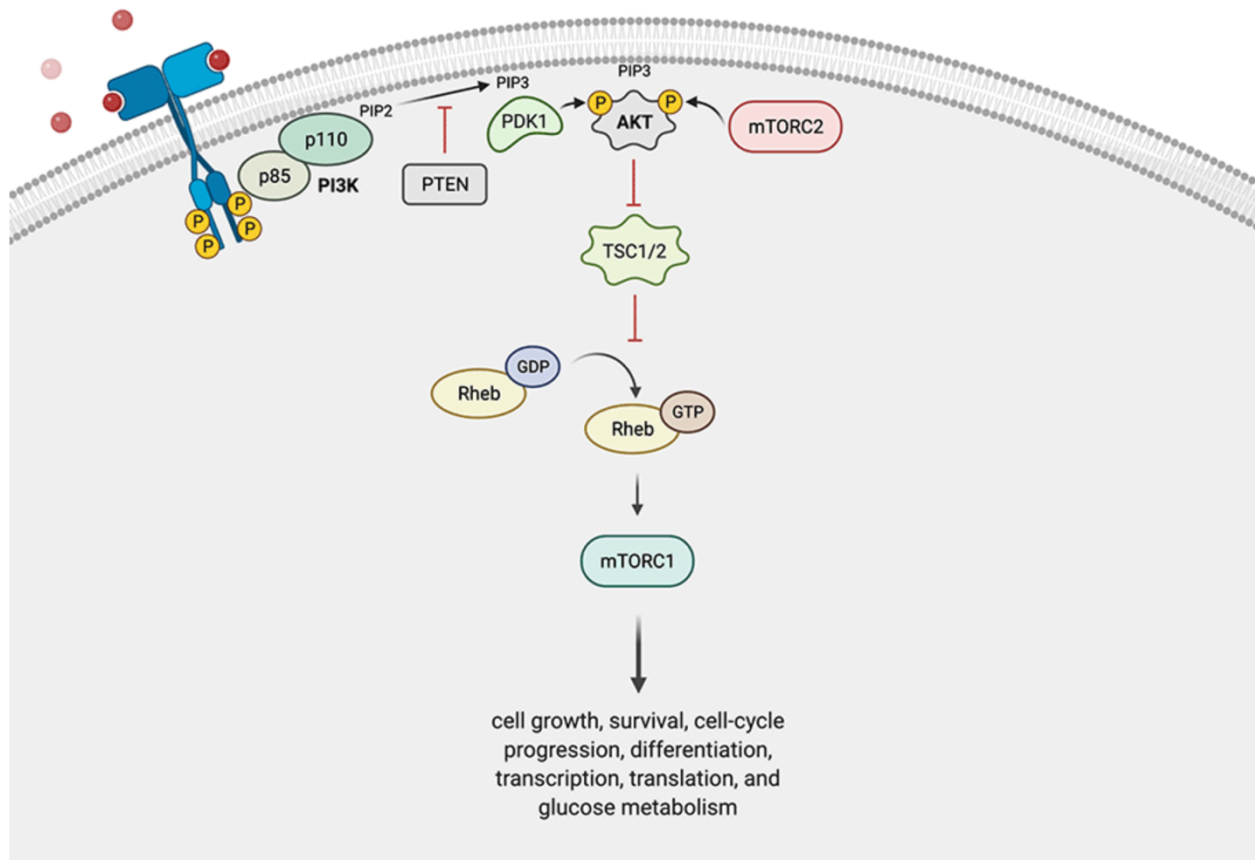
Figure 5. The AKT Pathway – Following generation of the seconder messenger PIP3 by PIK3, both phosphoinositide-dependent kinase-1 (PDK1) and AKT are recruited to the membrane. The proximity of these kinases allows PDK1 to phosphorylate AKT at residue T308 of the activation loop. Subsequently, AKT is phosphorylated at residue S473 by mTORC2. This phosphorylation is necessary to full activate the kinase activity of AKT. AKT itself can phosphorylate several other substrates that play important roles in cell survival and growth (Castel et al, 2014).
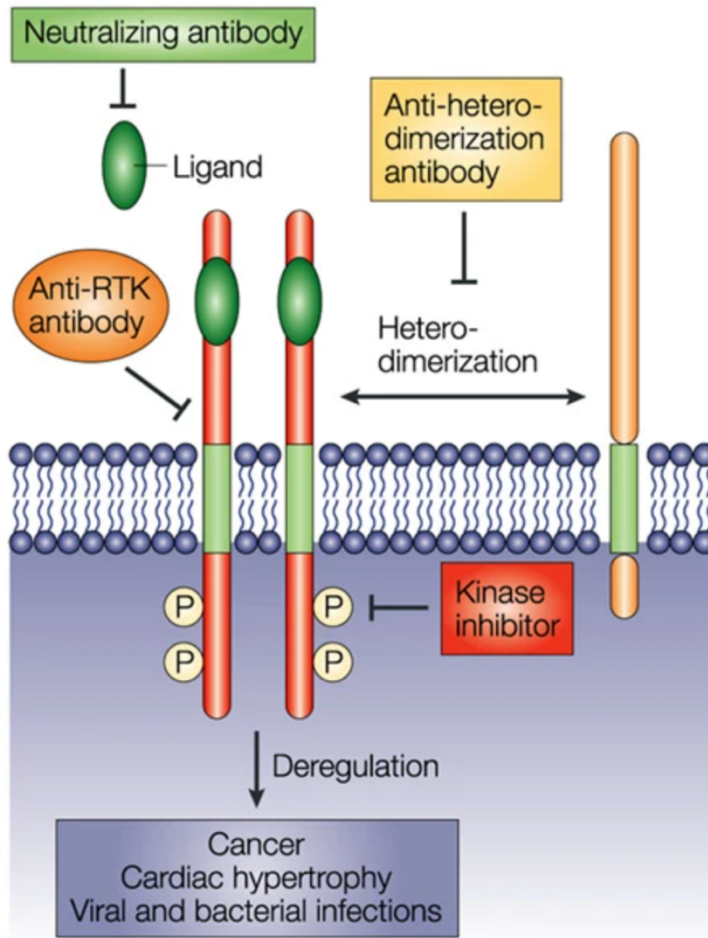
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Figure 6. Targeting RTKs as cancer treatment – Neutralizing antibodies block the activity of RTK ligands, RTK-targeted antibodies target overexpressed receptors or receptor dimerization, small molecule inhibitors, such as toceranib, interfere with RTK signal transduction, oden by blocking ATP binding sites. (Cschwind et all, 2004).
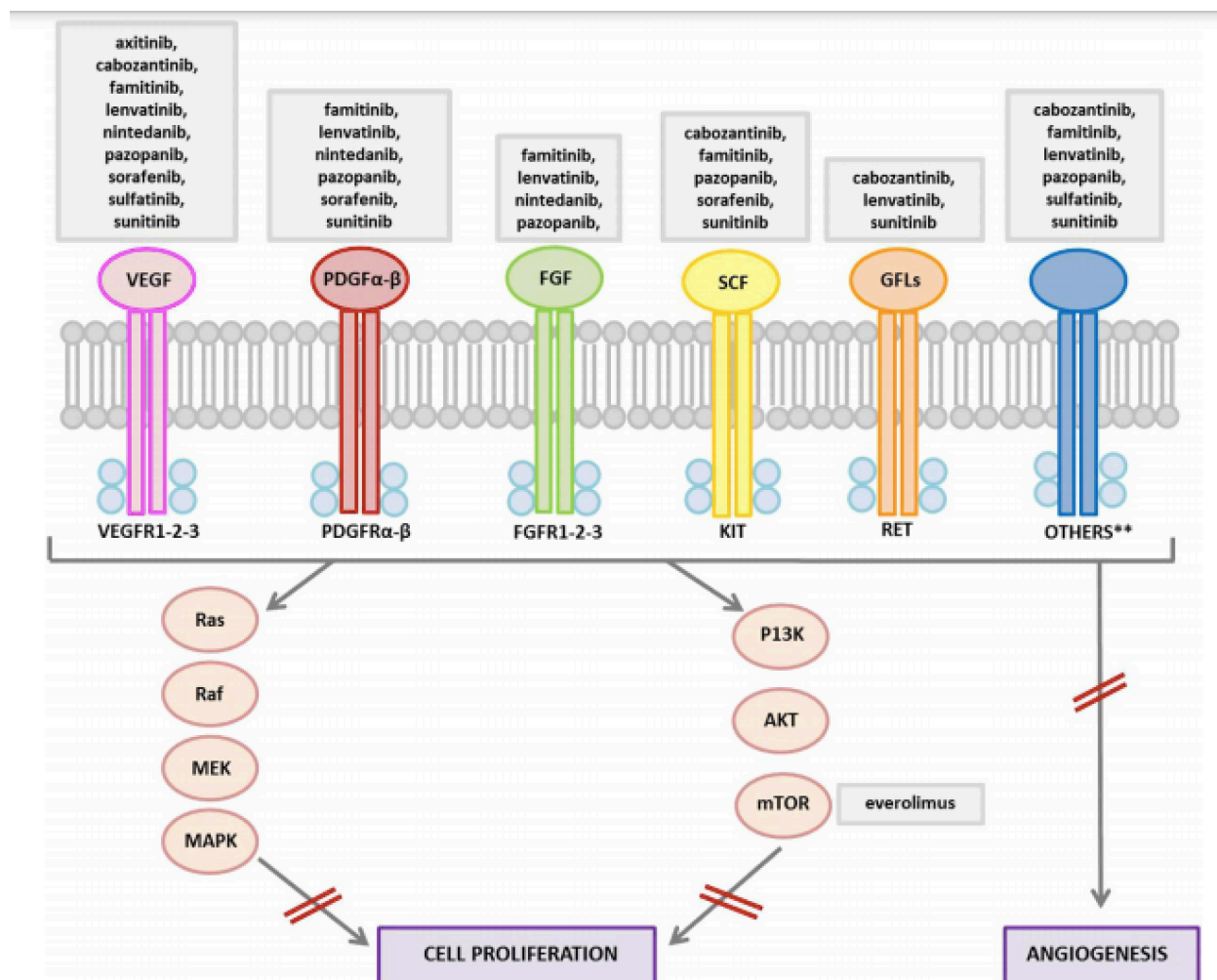
Figure 7. Targets of toceranib and their role in cancer – tyrosine kinases of all types are connected to the proliferation and survival pathways MAPK and AKT/mTOR. Mutations or aberrations in these receptors that lead to over-stimulation of the downstream signaling pathways can lead to the development of cancer. Multiple small molecule inhibitors have been created in human medicine to halt the activity of these receptors and their downstream signals. The targets of toceranib include VEGFR, PDGFR, and SCF. (Grillo et al, 2018).
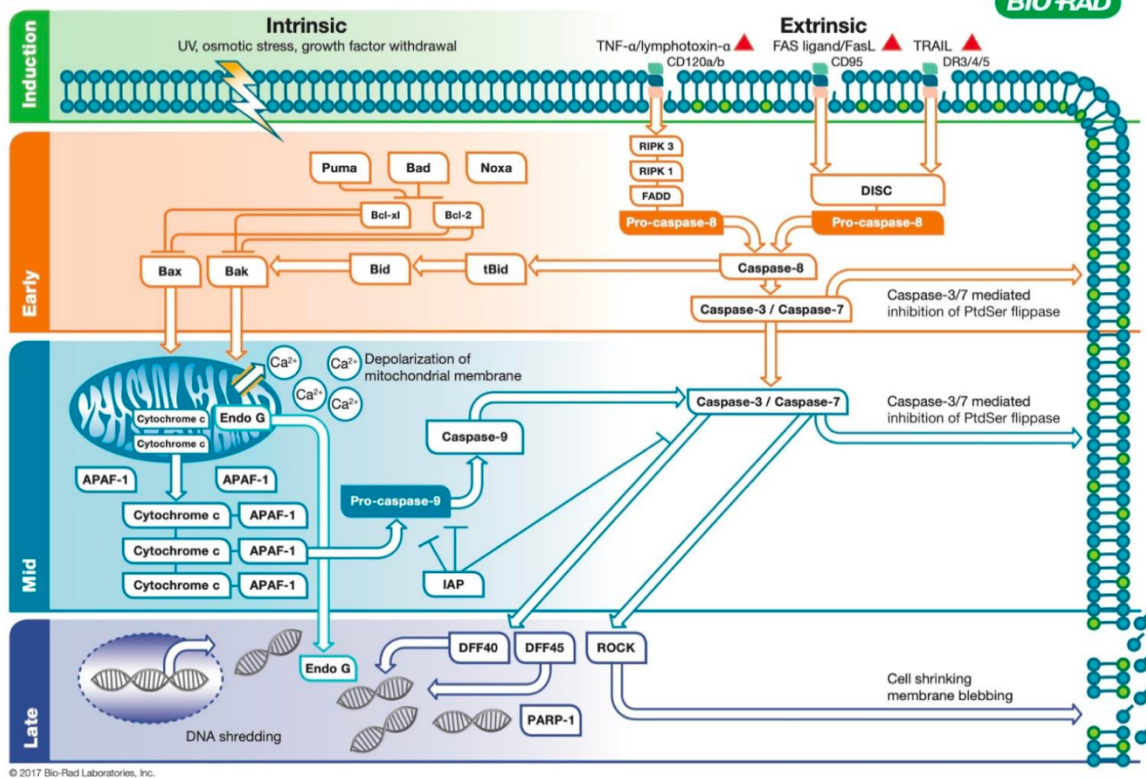
Figure 8. Stages of apoptosis. PAC-1 leads to activation of procaspase-3 to caspase-3, which is an effector caspase with activity in the mid stage of apoptosis. PARP activity is seen in the late stage of apoptosis. (BioRad, 2017).
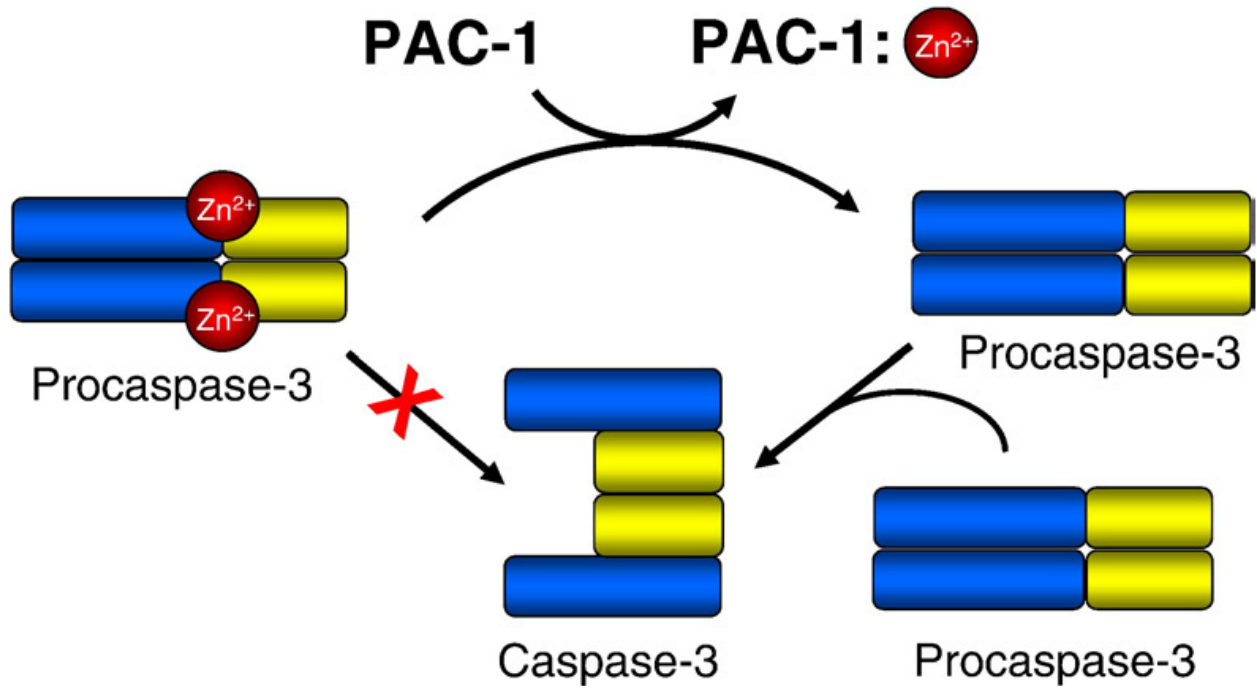
Figure 9. Procaspase-activating compound-1 – PAC-1 chelates zinc molecules that are normally inhibitory to the activation of procaspase-3 to caspase-3.
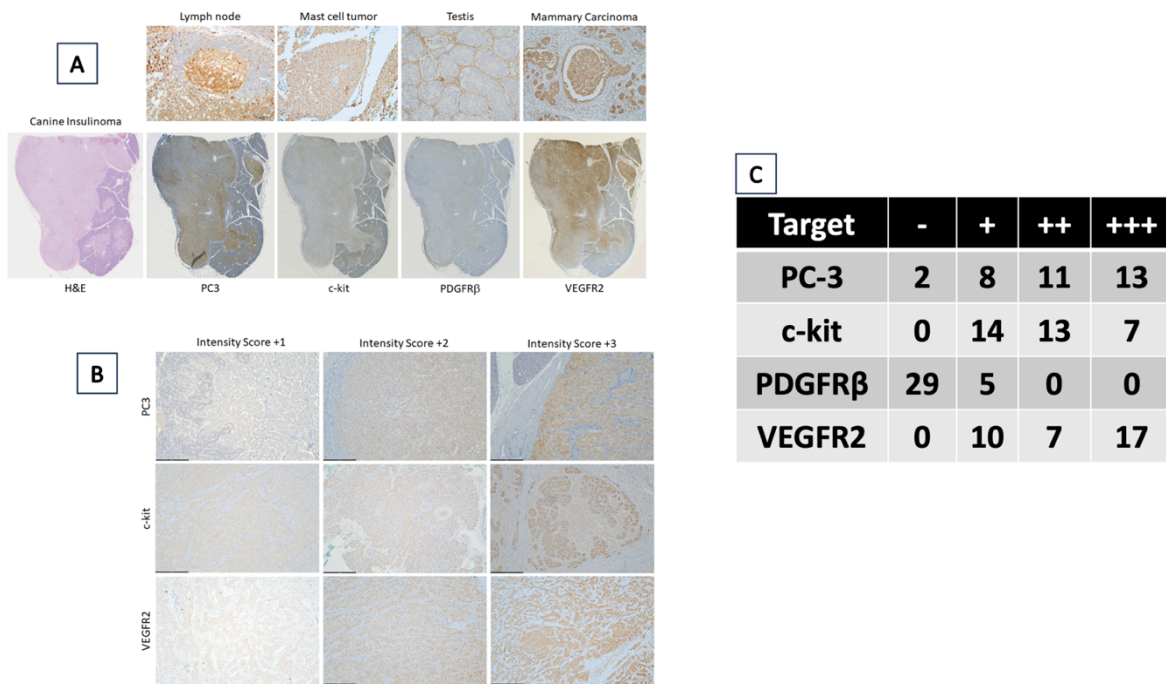
Figure 10. Presence of treatment targets in canine insulinoma – (A) Immunohistochemistry was used to evaluate for the protein expression of the targets of toceranib (C-kit, PDGFR beta, and VEGFR2) and PAC-1 (Procaspase-3). (B) Expression was scored from 0-3 based on intensity of staining. (C) PDGFR beta showed the weakest staining across the samples, c-KIT showed moderate staining, and VEGFR2 and PC-3 had the most robust staining.
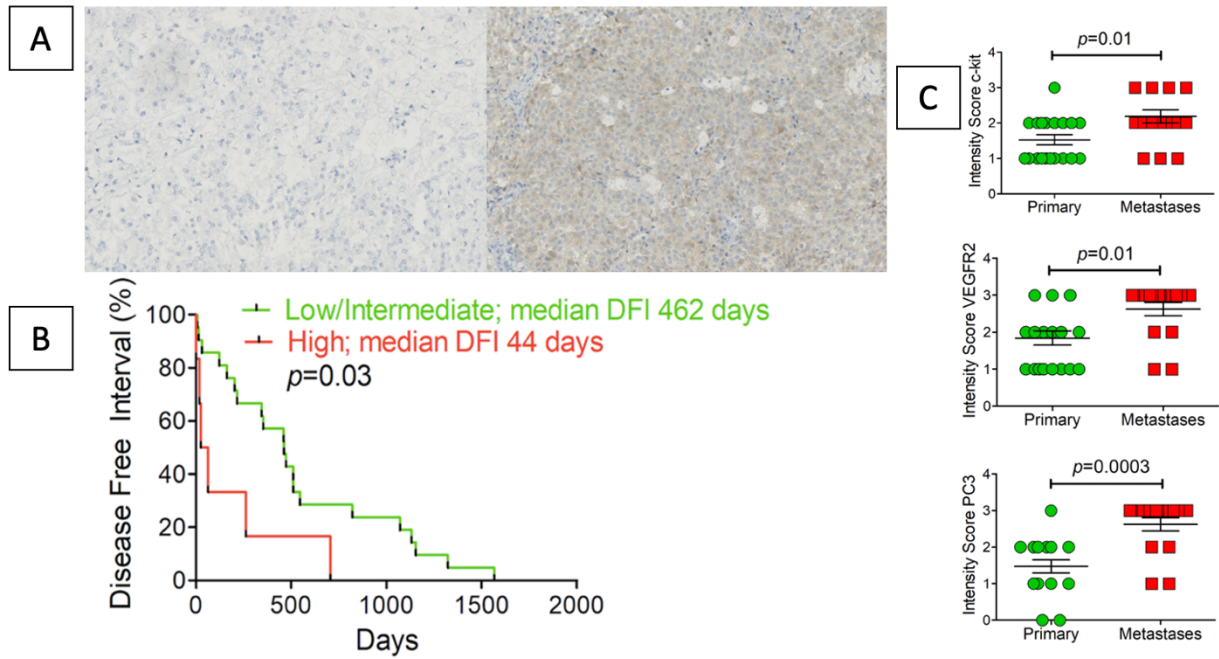
Figure 11. Expression of targets between primary and metastatic lesions, and prognostic significance of PC-3 expression – (A) PC-3 expression between a primary lesion (left) and a matched metastatic lymph node (right). (B) PC-3 staining intensity was significantly negatively correlated with outcome in evaluation of an outcome-linked tissue array. (C) c-KIT, VEGFR2, and PC-3 were more robustly expressed in metastatic lesions compared to primary lesions
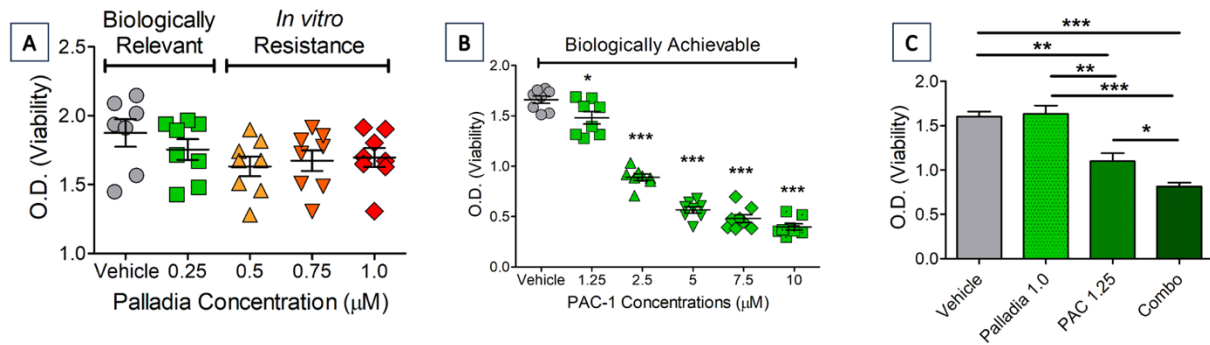
Figure 12. Canine insulinoma cell survival with toceranib and PAC-1 treatments – (A) No significant difference in cell death with increasing doses of toceranib. 0.25 uM is what is biologically achievable in dogs. (B) Dose-dependent cell death with PAC-1 treatment. (C) No difference in cell density with high dose toceranib. Significant decrease in cell density with low dose PAC-1 treatment. Significant decrease in cell density when low dose PAC-1 combined with high dose toceranib treatment.
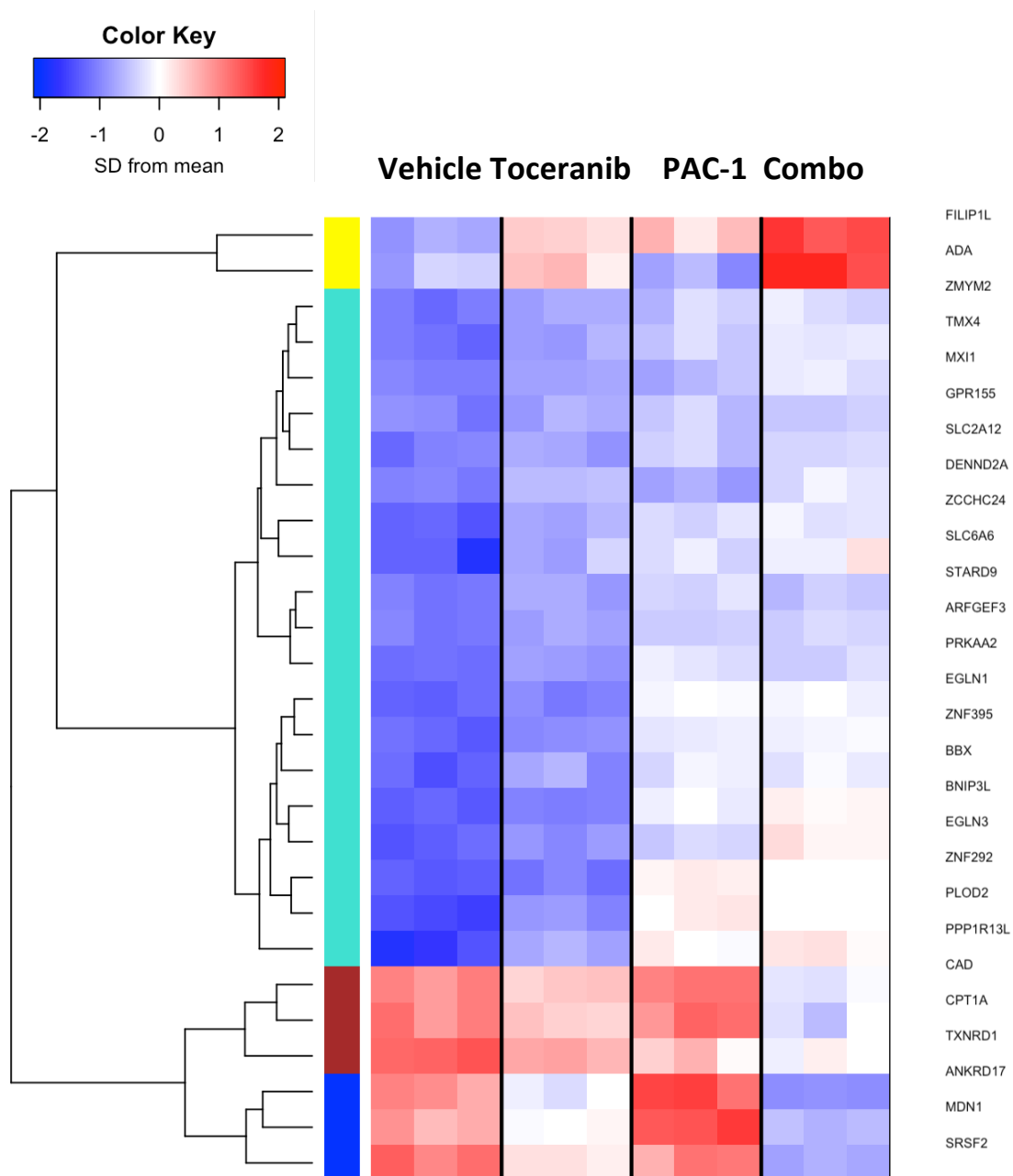
Figure 13. Transcriptional responses with Palladia and PAC-1 treatments – 27 differentially expressed genes were identified. The genes FILPIL, ADA, CAD, CPT1A, TXNRD1, ANKRD17, MDN1, and SRSF2 were transcriptionally changed in synergistic ways with combination treatment compared to single agent treatments.
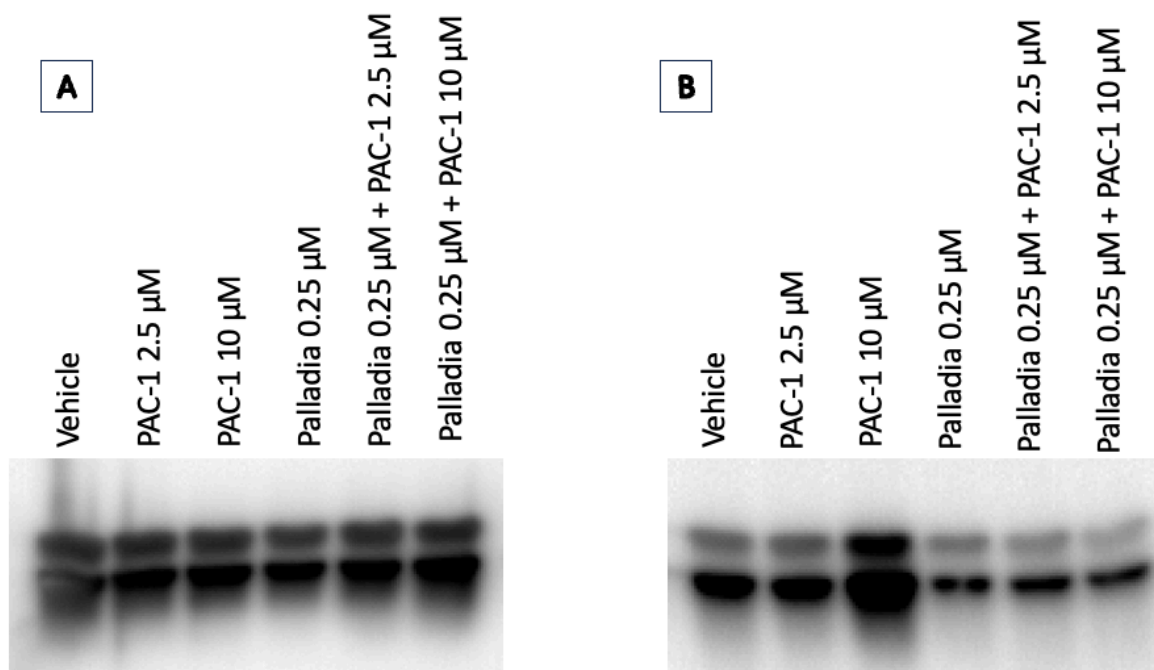
Figure 14. Erk phosphorylation with Palladia and PAC-1 treatments – (A) Erk is expressed similarly between all samples after 24 hour treatment. (B) Phospho-Erk blot. Erk is phosphorylated similarly between low dose PAC-1 treatment and control, however more Erk is phosphorylated with high dose PAC-1 treatment after 24 hours. Toceranib treatment led to a decrease in Erk-phosphorylation regardless of the presence of low or high dose PAC-1.
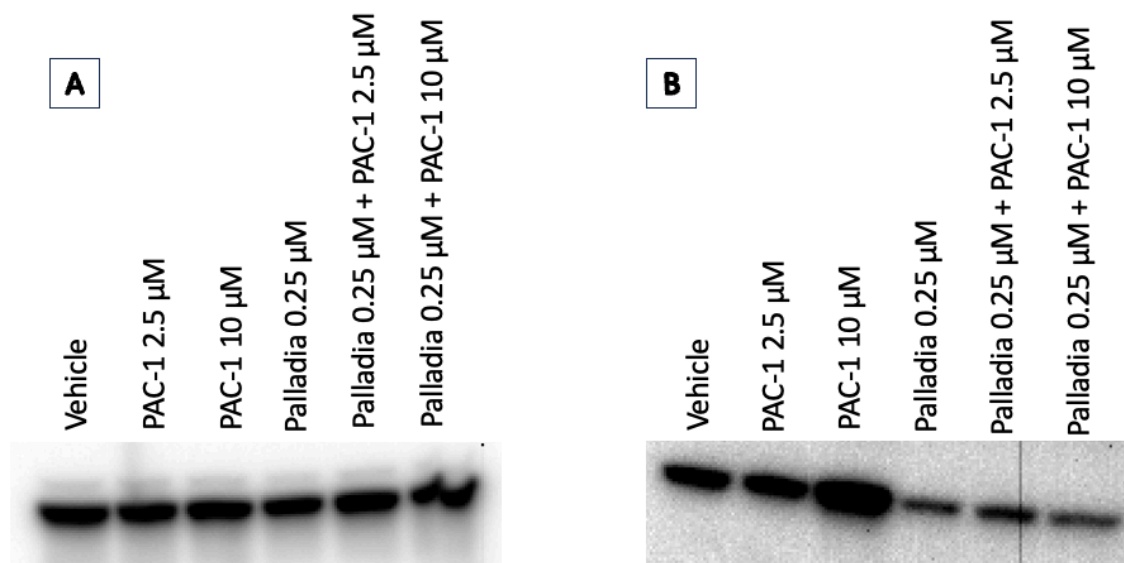
Figure 15. AKT and phosphorylated AKT with toceranib and PAC-1 treatments - (A) AKT is expressed similarly between all samples after 24 hour treatment. (B) Phospho-AKT blot. AKT is phosphorylated similarly between low dose PAC-1 treatment and control, however more AKT is phosphorylated with high dose PAC-1 treatment after 24 hours. Toceranib treatment led to a decrease in AKT-phosphorylation regardless of the presence of low or high dose PAC-1.
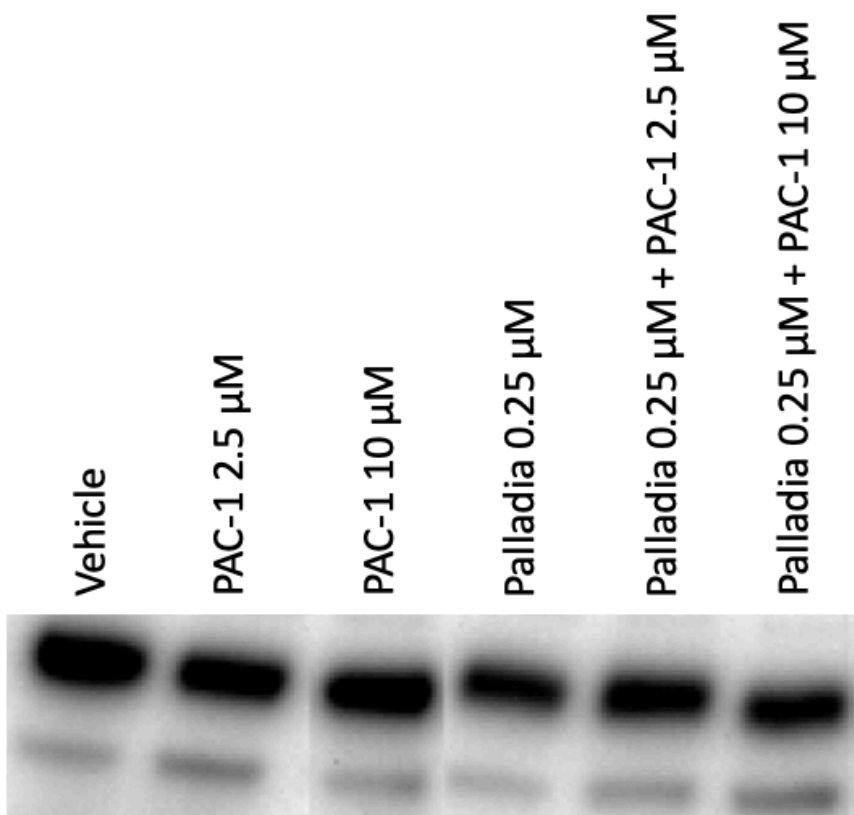
Figure 16. PARP cleavage with toceranib and PAC-1 treatments – PAC-1 demonstrated increased PARP cleavage compared to vehicle treated cells. Toceranib did not cause any meaningful PARP cleavage compared to vehicle treated cells. The combination treatments do not appear to have led to an increase in PARP cleavage compared to PAC-1 treatments.

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