INVESTIGATION OF THE BETA 2 ADRENERGIC RECEPTOR (B2-AR) PATHWAY IN CANINE HEMANGIOSARCOMA

BY

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THESIS

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ABSTRACT

Canine hemangiosarcoma is a highly metastatic cancer arising from vascular endothelial cells. It is one of the most aggressive canine cancers and most dogs die from this disease within a few months of the diagnosis. Despite advancements in veterinary oncology, there has been minimal improvement in the overall survival time even with standard treatment, which includes surgery and chemotherapy. Propranolol, an oral drug originally developed for the treatment of cardiovascular diseases, has been successfully used for the treatment of infantile hemangioma which is a benign neoplasia of vascular endothelial cells. Propranolol blocks adrenergic receptors, which would otherwise bind to catecholamines responsible for the “stress signal” leading to many physiologic changes. Stress has been implicated in many models of carcinogenesis and tumor progression. Given the relationship between stress and cancer, as well as similarities between canine hemangiosarcoma and infantile hemangioma, we sought to investigate the presence of the beta 2 adrenergic receptor and the effects of propranolol in canine hemangiosarcoma. We demonstrated the presence of the beta 2 adrenergic receptor via immunohistochemistry in all 18 tissue samples of spontaneous canine splenic hemangiosarcoma and in canine hemangiosarcoma cell lines Fitz and DEN, cell line receptor expression was also confirmed with Western blot. Src, a possible intermediary downstream protein involved in adrenergic signaling was also investigated and both Fitz and DEN exhibited the presence of the Src protein in Western blot. Activation of this pathway would involve phosphorylation of Src upon catecholamine binding to the receptor, which was investigated through Western blot. Fitz and DEN exhibited basal phosphorylation of Src and after treatment with norepinephrine, and Fitz exhibited greater phosphorylation (23% increase compared to basal control) after 60-minute exposure to the agonist. Fitz cells were pretreated with a biologically achievable dose of
propranolol followed by the agonist, and a modest decrease in phosphorylation was observed (11% decrease compared to basal level). Further investigation into the biological effects of propranolol in Fitz and DEN revealed a decrease in VEGF secretion, increase in proliferation and decrease in cell migration. Reduction in VEGF secretion was evaluated via ELISA and it was present at propranolol doses greater than 10 µM for DEN and Fitz, achieving a maximum reduction of 21% in DEN and 44% in Fitz compared to untreated cells. Cell proliferation was measured through MTS assay, which revealed an increase in cell proliferation only in Fitz cells treated with 0.1 µM of NE (25% increase) and cells treated with 0.1 µM of propranolol (30% increase). Cell migration was evaluated with a scratch assay and was decreased only when cells were treated with propranolol at a high dose (100 µM). Taken together, the findings of this study show that beta 2 adrenergic receptors are expressed by canine hemangiosarcoma, Src may be involved in the downstream signaling from the receptor and blockade of the receptor leads to mild to moderate effects in cell angiogenesis, proliferation and migration.
For my family,

Luiz, Thais, Rodrigo and Victoria Portela

for never letting me feel alone

and for always believing in me
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CHAPTER 1

INTRODUCTION

Canine hemangiosarcoma (cHSA) is a malignant neoplasm of endothelial cells that affects many species, being more frequent in dogs [1-3]. This disease is highly metastatic and the prognosis is poor, with a median survival time of about 6 months with standard therapy, which involves surgery and chemotherapy treatment [4]. Limited advancements have been made to improve survival time therefore novel therapies are needed. Drug repositioning, which uses known drugs for alternative purposes (treatment of new diseases), is a strategy that can be pursued with the advantage of being of lower cost (pharmacokinetics and pharmacodynamics are already known; drugs already in the market can be selected for their cost) and known tolerability. Recently, propranolol has received attention for its effects on the treatment of infantile hemangioma (IH), a benign neoplasm of endothelial cells. It was accidentally discovered while treating infants with hypertension that propranolol (a β-adrenergic receptor antagonist) has activity against IH, even in treatment-resistant cases [5]. This prompted many studies, mostly clinical [6-11], looking at the effects of propranolol as an anti-neoplastic drug. Since both cHSA and IH are tumors of endothelial cell origin, we developed an interest in investigating the effects of propranolol in cHSA. Parallel to IH and the recent propranolol discovery, there is also a link between stress and cancer. Stress is physiologically described as the binding of catecholamines to the adrenergic receptors, which mediate the “fight-or-flight” response necessary for survival of an organism. An interesting hypothesis is that this adrenergic signaling may also be functioning to maintain survival and/or progression of neoplastic cells exposed to the same catecholamines released during stressful situations, which are common during diagnosis and
treatment of cancer. To further support this hypothesis, studies linking breast cancer survival and β-blockers have been published suggesting that inhibition of the β-adrenergic signaling pathway can reduce breast cancer progression, recurrence, metastasis and mortality [12, 13]. Many other studies supporting the role of the β-adrenergic pathway in cancer development have been described and will be discussed in the next section.

We hypothesized that cHSA expresses the receptors necessary for adrenergic signaling (β2-AR), an intermediary kinase (Src) is involved in the signaling pathway downstream from the receptor and propranolol treatment would lead to decrease in cell proliferation, angiogenesis and migration.
CHAPTER 2

LITERATURE REVIEW

2.1. Stress and tumorigenesis

The process of tumorigenesis and metastasis has been extensively studied in the scientific community. Many environmental components have been implicated in cancer development and new compounds with carcinogenic activity are frequently being discovered. Stress is one environmental component that may influence the carcinogenic process and potentiate tumorigenesis. Stress is defined as “A state of mental or emotional strain or tension resulting from adverse or very demanding circumstances” and “Pressure or tension exerted on a material object” [14, 15]. In modern life, people are subjected to stress from many different aspects. Stressful experiences include physical stressors such as pathogens and toxins, and psychological stressors such as major life events, trauma, abuse, or factors related to the environment in the home, workplace or family [16].

In 1936, Hans Selye defined stress physiologically as the state in which the sympathoadrenomedullary system and the limbic-hypothalamic-pituitary-adrenal axis are co-activated [16, 17]. From a biological standpoint, it is known that stress, whether physical or mental, culminates in the release of hormones named catecholamines, which mediate the “fight-or-flight” responses causing activation of the sympathetic nervous system [19]. The most abundant catecholamines are epinephrine (E), norepinephrine (NE) and dopamine, which are released from the chromaffin cells of the adrenal medulla and from neuro-muscular junctions [19, 20]. In humans, acute stress can elevate the levels of norepinephrine and epinephrine by
greater than 10-fold in only a few seconds, although chronic stress can lead to fluctuations on the catecholamine levels as well [19, 20].

Cancer patients are subjected to stress from different aspects, from dealing with the acceptance of a terminal disease (sometimes associated with anxiety and depression) to the homeostatic effects of stress on inflammation, immunocompetence, and cancer initiation or progression [19, 21-28].

Evaluating stress in canine patients is difficult, especially the psychosomatic aspects. Physiologically there have been attempts at quantifying mediators of stress signaling. Increase in plasma epinephrine and norepinephrine levels in dogs was documented in a study that compared levels of these catecholamines in groups subjected to environmental stress of blood collection immediately after arrival to the laboratory versus 15 minutes after arrival, indicating the rapid fluctuation of these hormones under different stress conditions [29]. Levels of catecholamines in dogs subjected to various stressful stimuli were measured and in the literature they ranged around 65 to 239 pg/mL for epinephrine, 145 to 524 pg/mL for norepinephrine and 20-437 pg/mL for dopamine [14, 29]. The release and degradation of catecholamines is variable and thus the concentration of these hormones can differ significantly across different tissues and within the circulating blood [2]. In humans, the difference can be in the order of nanomolar for circulating NE versus micromolar concentrations in the tissue microenvironment [30].

2.2. **Adrenergic pathway**

Catecholamines mediate adrenergic signaling via receptors in target cells. Receptors include α1A,B,D; α2A,B,C; β1,β2 and β3 and are present in a wide variety of cells and tissues,
including central nervous system (CNS), lungs, liver, pancreas, kidney, adrenal glands, breast, ovary, prostate, vasculature, bone marrow and cells of the immune system (macrophages, mast cells, lymphocytes) [19, 20]. The expression of β1 and β2 adrenergic receptors (AR) has been extensively studied in the cardiovascular system, with high expression occurring in cardiac myocytes and vascular smooth muscle cells [31]. These receptors belong to a family called G-protein coupled receptors (GPCRs) [32], which contain seven transmembrane “serpentine” segments with the N-terminal facing the extracellular space and the C-terminal immersed in the cytoplasm. When the ligand binds to the extracellular domain of the receptor, the receptor undergoes a conformational change, which will then activate the G-protein located in the intracellular space. The G-protein is composed of a α, β and γ units, with the α and γ subunits having lipid tails that covalently attach the protein to the cell membrane. In the absence of ligand binding, the resting G-protein’s alpha subunit is bound to GDP (guanosine diphosphate). When the signal is initiated by catecholamine binding, the receptor undergoes a conformational change and GDP is replaced by GTP (guanosine triphosphate) in the alpha subunit of the G-protein. This protein detaches from the transmembrane receptor and moves on to activate other downstream proteins (Fig. 2.1). Inactivation of the protein occurs when phosphatase activity removes a phosphate group from GTP transforming it back to GDP form [33].

Beta 2 adrenergic receptors (β2-AR) are the focus of this project due to its demonstrated expression in different human vascular tumors [34]. These receptors activate adenylyl cyclase which in turn will catalyze the conversion of ATP to cyclic AMP (cAMP). Cyclic AMP can act as a secondary messenger itself or it can bind to other downstream proteins, such as protein kinase A [33, 35, 36]. Protein kinase A can phosphorylate other target proteins including cAMP-responsive element binding protein/activating transcription factor (CREB/ATF) and β-adrenergic...
receptor kinase (BARK) [19]. Upon continuous stimulation, the receptor eventually becomes inactivated by the inability to bind to G-proteins. That is achieved by phosphorylation of the intracellular portion of the receptor by BARK, and the phosphorylated protein binds to β-arrestin, which blocks the connection between the receptor and the G-proteins (Fig. 2.2). β-arrestin also can function as an adaptor protein, and is this way that it recruits Src to become bound to the β2-AR [37]. Furthermore, it has also been shown that cAMP/PKA mediates NE-induced activation of Src [38].

Src is a non-receptor tyrosine kinase ubiquitously expressed in many tissues, and plays an important role in transmitting signals from the cell surface to the nucleus via phosphorylation of tyrosine residues of intracellular proteins [39]. Src is involved in the maintenance of normal cell homeostasis including cell proliferation, survival, maintenance of cytoskeleton, cell adhesion and motility [38, 39]. Src is overexpressed in many human malignancies, including colorectal, breast, prostate, pancreas, head and neck carcinoma, lung carcinoma, glioma, melanoma and sarcomas [38, 40, 41]. A recent study has demonstrated relationship between increased phosphorylation of Src caused by NE stimulation and tumor growth and progression in ovarian carcinoma murine models, assessed as number of nodules and tumor weight. Propranolol, a non-selective β-blocker (described in detail later in this chapter) successfully counteracted the effects of stress and of adrenergic agonists (isoproterenol and terbutaline) evidenced by decreased tumor weight and number of nodules in mice, which was similar to control (non-stressed or non-treated mice) [38]. Src has been also implicated in NE-stimulated vascular endothelial growth factor (VEGF) production by adipocytes [42, 43]. VEGF is a cytokine known to stimulate angiogenesis in both normal and neoplastic tissue. In addition, it was demonstrated that Src is present in canine HSA cell lines and its reduced expression was linked to a decrease in cell viability [44].
2.3. **Adrenergic pathway and molecular pathogenesis of cancer**

2.3.1 **Breast cancer**

The β2-AR and its signaling pathways have been extensively studied in human oncology. In breast cancer, preclinical models suggest that this pathway may influence breast cancer progression through: 1) increased tumor cell survival after exposure to chemotherapeutic agents; 2) increased breast cancer cell proliferation; 3) altering the tumor microenvironment in angiogenesis and inflammatory response [27].

At therapeutic levels, β-blockers do not seem to be directly cytotoxic; rather the β-AR blockade may inhibit catecholamine signaling that normally would lead to pro-tumorigenic effects (cell survival, proliferation, migration, angiogenesis) [45]. Therefore, research efforts are now focused on the ability of β-blockers to inhibit cancer progression in already established cancers. In one study using an orthotopic mouse model looking at the effect of chronic stress in breast cancer progression, mice were injected with luciferase-transfected breast cancer cells into the 4th mammary pad and physically restrained for 2 hours daily for 20 days, while the control group was not subjected to stress. The results showed a 37-fold increase in metastasis to the lungs and 67% increased metastasis to the lymph nodes in the stressed group compared to control [30]. In a separate experiment, mice were pre-treated with propranolol prior to tumor inoculation and stress stimuli. There was an increased expression of β2-AR in tumors in propranolol-treated animals and propranolol significantly decreased the metastatic burden in stressed animals. There was no change in metastasis for non-stressed animals, and treatment did not affect primary tumor growth in either group [30]. This study provided some objective evidence of the role of adrenergic signaling and breast cancer progression.
Catecholamines alter the cytokine profile of the bone microenvironment and promote the incidence of metastatic colonization by breast cancer cells. Receptor activator of nuclear factor kappa-B ligand (RANKL) is a protein involved in bone remodeling as well as dendritic cell maturation and is secreted by osteoblasts in response to sympathetic activation. RANKL stimulates breast cancer migration and bone colonization and is recognized as a crucial factor for cancer cell motility in addition to its well-established role in tumor-induced osteolysis [46]. This pathway was inhibited by propranolol therapy [47]. Activation of the sympathetic system promoted bone metastasis in a mouse model of breast cancer. Stimulation of the β2-AR induced RANKL expression in bone marrow osteoblasts and increased migration of metastatic MDA-MB-231 mammary carcinoma cells in vitro. Mice models of bone metastasis subjected to chronic stress had sympathetic activation blocked by propranolol. Sympathetic nerves inhibit osteoblast proliferation and regulate hematopoietic stem cell proliferation, survival and trafficking [48, 49]. Norepinephrine released from the sympathetic nerves stimulate the formation of osteoclasts [50].

Perhaps one of the most commented serendipitous discovery was that women who took β-blockers (specifically propranolol) for cardiovascular disease were found to be significantly less likely to die of their cancer, and less likely to present at higher stages of the disease, compared to women who did not take propranolol [12]. Another study showed a 57% reduced risk of metastasis and a 71% reduction in breast cancer mortality after 10 years [13]. Further evidence also showed that β-blocker intake was associated with improved relapse-free survival but not overall survival in patients with breast cancer, including triple-negative tumors which are tumors negative for epidermal growth factor receptor (HER2), progesterone receptor (PR) and estrogen receptor (ER) [51]. Relapse is more frequent among triple-negative breast cancer patients, especially within the first 3 years of diagnosis, compared to patients with ER
positive tumors [52, 53]. High β2-AR expression was found in tumors containing estrogen receptors that were hormonally positive, and patients carrying these tumors had a worse prognosis five years later, coincidentally with the discontinuation of tamoxifen therapy. The prognosis was good within the first five years, hinting at a possible advantage of combination therapy with propranolol, which could have an impact in breast cancer survival [54].

Despite some encouraging published evidence on the benefits of propranolol in cancer patients, there is still some controversy. There are studies suggesting an increased risk or no survival advantage of various cancers including colon, lung, breast and prostate cancer in patients taking β-blockers [55-57]. However, patients taking β-blockers for various medical reasons may have already chronically elevated levels of catecholamines, which would promote cancer progression and worse outcome in cancer-bearing patients. In that sense, the chronically elevated NE would be a higher risk than the propranolol itself [58]. A large meta-analysis failed to demonstrate strong evidence that β2-AR blockers promote tumor progression [59]. In summary, these studies demonstrate that elevated levels of catecholamines as well as blockade of the β-adrenergic receptor pathway by propranolol may have some impact in breast cancer progression as well as patient survival.

2.3.2. Pancreatic carcinoma

In pancreatic carcinoma, β2-AR was expressed in cell lines. NE promoted increased proliferation of these cells in a dose-dependent fashion, also promoted S-phase cell cycle shift and decreased cells in the G1/G2 phase. Migration was also increased with NE treatment as well as increase in phosphorylation of p38 (MAPK pathway, important in cell proliferation), which was blocked by treatment with propranolol [60]. In another study, invasiveness and angiogenic
potential of pancreatic carcinoma cell lines were assessed by measurement of matrix
metalloproteinases (MMPs) MMP-2, MMP-9 and VEGF. Norepinephrine promoted the
invasiveness of one of the cell lines in a concentration-dependent manner, and NE increased the
expression of MMP-2, MMP-9, and VEGF. These effects were inhibited by propranolol [61].
Furthermore, propranolol significantly suppressed cell invasion and proliferation in comparison
to β1-adrenergic specific antagonist metoprolol. Treatment with β2-adrenoceptor antagonists
inhibited activation of transcription factors nuclear factor κB (NF-κB), activator protein 1 (AP-1)
and cAMP response element binding protein (CREB). β2-adrenoceptor antagonists also
significantly altered VEGF, cyclooxygenase-2 (COX-2), MMP-2 and MMP-9 expression
confirming previous findings. The β2-adrenergic antagonists suppressed invasion and
proliferation by inhibiting both cAMP/PKA and Ras, which regulate activation of the MAPK
pathway and transcription factors, such as NF-κB, AP-1 and CREB, as well as expression of
their target genes, MMP-9, MMP-2 and VEGF. β1-adrenergic antagonists suppressed invasion
by inhibiting only the cAMP/PKA pathway [62]. Proliferation and migration of human
pancreatic ductal carcinoma is stimulated in vitro by β-AR and subsequent cAMP-dependent
signaling, which in turn leads to cAMP-dependent release of epidermal growth factor, and PKA-
dependent release of VEGF. These effects were also blocked with propranolol treatment [63].
Another study using isoproterenol (a non-selective β-AR agonist) significantly increased cell
proliferation of a pancreatic ductal carcinoma cell line in a dose-dependent manner, with
concomitant activation of ERK/MAPK signal pathway as well as increased levels of
phosphorylated ERK [64, 65]. It has been previously demonstrated that β2-AR causes activation
of the extracellular-signal regulated kinase 1/2 (ERK 1/2).
In vivo, mice carrying xenograft tumors had enhanced tumor growth caused by isoproterenol, which was suppressed by propranolol [64]. Taken together, these studies suggest that blocking the adrenergic pathway using propranolol may have some impact in pancreatic carcinoma progression.

2.3.3. Ovarian carcinoma

Immunohistochemistry demonstrated stress-induced increases in levels of basic fibroblast growth factor (bFGF), MMP-2 and MMP-9 proteins in ovarian carcinoma. Catecholamines have previously shown to promote VEGF production by ovarian cancer cells in vitro [66, 67].

Chronic behavioral stress resulted in higher levels of tissue catecholamines, greater tumor burden and more invasive pattern of ovarian cancer growth in a mouse model; effects which were mediated by β2-AR activation of PKA signaling in the cancer cells [68]. Another interesting study by Thacker et al used periodic physical restraint to stimulate chronic stress in nude mice inoculated with human ovarian carcinoma cells in the peritoneal cavity. In mice receiving 0, 2 or 6 hours of immobilization daily for 21 days, the number of tumor nodules increased by 259% in the 2 hr stress group and 356% in the 6 hr stress group compared to unstressed mice. Mean tumor weight increased 242% in the 2 hr stress group and 275% in the 6 hr group compared to unstressed mice. Social isolation as an alternative stressor also showed a 187% increase in tumor weight and a 255% increase in nodule count compared with group-housed control animals. Stressed animals and non-stressed mice treated with isoproterenol and terbutaline (β-agonists) had both significantly enhanced mean vessel density (MVD) counts (a measure of angiogenesis [69]) and that effect was blocked by propranolol. This was accompanied by significant elevation of VEGF protein and mRNA within tumor tissue.
In another model investigating the effects of surgical stress in tumor development, mice were injected with ovarian carcinoma cells and 4 days later subjected to either a laparotomy procedure (larger incision, more tissue handling and stress) or a laparoscopy procedure (less invasive, less stress). Mice in the laparoscopy group had significantly lower tumor weight compared to mice in the laparotomy group. In the immediate postoperative period, serum levels of VEGF and MMP-2 were significantly lower in the laparoscopy group as well, further supporting the mediators of stress-induced tumor progression [70]. Lee et al. had also demonstrated previously that surgery significantly increased MVD and VEGF expression, which were blocked by propranolol treatment in mice injected with ovarian carcinoma cell lines [71]. The stress of surgery is suggested to facilitate the post-surgery growth of pre-existing micrometastases and small residual tumors [72-74]. Taken together, these studies indicate that psychosomatic and surgical stress, both present in cancer patients, are linked to tumor growth and angiogenesis and propranolol appears to ameliorate these effects.

2.3.4. Prostatic carcinoma

Many studies have also been performed investigating the role of β2-AR in prostatic carcinoma. Among the studies investigating possible molecular pathways involved in β blockade and prostatic carcinoma, there appears to be a relationship between β2-AR and histamine, as histamine augmented β2-AR-induced cAMP accumulation independently of known histamine receptors [75]. β2-AR activation also promoted prostate cancer cell proliferation and cell migration through increasing cellular cAMP and ERK 1/2 activation in another study, where it also demonstrated the involvement of β-arrestin in this process (β-arrestin participates in agonist-mediated desensitization of G protein-coupled receptors). The formation of β-arrestin 2/c-Src
complex was a key factor in this process [76]. An experiment was performed where PC-3 xenografted mice were implanted with a norepinephrine-releasing micropump which subsequently led to a 1.6 fold increase in tumor metastasis compared to control [77]. In addition, behavioral stress activated β2-AR signaling and led to inhibition of apoptosis and accelerated cancer development in mice. The effects of stress were prevented with treatment using a β2-AR selective antagonist [78].

β2-AR is a known activator of the androgen receptor, and this receptor is upregulated in androgen-independent prostatic carcinoma cell lines. Expression of this receptor is greater in malignant cells compared to benign hyperplasia or normal tissue [79]. One study showed that NE drives metastasis of PC-3 cells in BALB/C3 nude mice and this process is inhibited by β-blockers [77]. Loss of BARK-1 in a group of patients with high-grade prostatic carcinoma could indicate a possible pathway of prostate cancer development involving β2-AR signaling [80]. BARK-1 is a serine/threonine kinase that desensitzes the receptors from catecholamine over-stimulation (negative feedback via recruitment of beta-arrestin) [81]. In another study, β-blocker use was not associated with increased risk of prostatic carcinoma development or overall mortality, however a subgroup of men treated with androgen deprivation therapy and a β-blocker had a fivefold reduced prostatic carcinoma specific mortality [82].

In summary, these studies indicate that prostatic carcinoma express the β2-AR, β2-AR activation promote prostate cancer cell proliferation and cell migration, mice exposed to increased levels of catecholamines have increased metastasis and men treated with β-blockers may have reduced cancer-related mortality.

2.3.5. Other malignancies
Stress has been suggested as a possible factor in human colorectal carcinoma development. In one study looking at the effects of chronic restraint stress in nude mice bearing human colorectal carcinoma xenografts, adrenergic signaling-dependent activation of ERK 1/2 promoted cell proliferation, and β-adrenergic antagonism inhibited proliferation and decreased phosphorylation of ERK 1/2 in vitro and in vivo. Norepinephrine and epinephrine enhanced colorectal carcinoma cell proliferation and viability in cell culture as well as tumor growth in mice. These effects were antagonized by propranolol and phentolamine (α-AR antagonist) [83]. The first epidemiological investigation of the effect of post-diagnostic β-blocker usage on colorectal cancer-specific mortality showed no association. There was some evidence of a weak reduction in all-cause mortality in β-blocker users which was in part due to the marked effect of atenolol on cardiovascular mortality [84].

Melanoma cell lines also expressed β1 and β2-ARs. Norepinephrine and E increased metalloprotease-dependent motility, released interleukin-6 (IL-6), interleukin-8 (IL-8) and VEGF. The effects of these catecholamines were inhibited by propranolol [85]. Interleukin-6 is involved in the host immune defense mechanism as well as the modulation of growth and differentiation in various malignancies [86]. Increased expression of IL-8 and/or its receptors has been characterized in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages, suggesting that IL-8 may function as a significant regulatory factor within the tumor microenvironment [87]. Expression of β-ARs had been previously identified in melanoma cell lines as well as human melanoma biopsies and NE upregulated production of VEGF, IL-8, and IL-6 in the cell lines [88]. Overall, the results from preclinical studies support the suggestion that β-blockers could provide a clinical benefit in melanoma progression however epidemiological studies are limited by sample size, which is also a limitation in breast, colorectal
and prostatic carcinoma studies [89]. In patients with melanoma, the β-blocker-treated group had an overall improved survival after a median follow-up of four years and for each year of β-blocker use, the risk of death was reduced by 38% [90]. Another study found that both β1 and β2-ARs are expressed in tissues from benign melanocytic nevi, atypical nevi and malignant melanomas and that expression was significantly higher in malignant tumors.

In an *in vitro* multiple myeloma study, propranolol IC50 values (concentration of propranolol required for 50% inhibition of proliferation) were decreased over time. There were also significant increases in caspase 3 activity, in apoptotic cell population, and a decrease in expression levels of Bcl-2 (anti-apoptotic protein) in response to propranolol treatment [91]. Caspase-3 is an executioner caspase protein, involved in the process of apoptosis (programmed cell death). Another study showed NE-induced secretion of VEGF in 3 multiple myeloma cell lines [92].

In conclusion, although the relationship of stress and tumor development/progression has been more extensively studied in a number of specific carcinomas (breast, ovarian, prostatic, colorectal, pancreatic), it appears to influence non-carcinoma malignancies as well (melanoma, myeloma). This illustrates how broad this research field is and indicates that propranolol may have the potential to be of benefit in the treatment of many other tumors.

2.4. **Propranolol**

Propranolol is an attractive potential anti-neoplastic therapeutic option since it is affordable, easily obtainable in the market and the pharmacokinetics have been established for humans [93] and dogs [94]. It is a non-selective β-blocker, available as propranolol
hydrochloride, under the brand name Inderal® in North America by AstraZeneca®. It has been mainly used for the treatment of hypertension, although other conditions reported include tachyarrhythmias [95], tachycardia, essential tremor [96, 97], migraine [98, 99], post-traumatic stress disorder [100-104], glaucoma [105], akathisia (restlessness) [106], schizophrenia (elevated levels of norepinephrine may play a causative role in the development of this disease) [107], adrenergic urticaria [108], treatment of burn patients [109-112] and anxiety [113, 114].

In human patients, receiving the diagnosis of cancer creates significant levels of emotional distress, with intrusive thoughts (emotional memories, flashbacks, nightmares, and intrusive images) being the most common manifestation among breast cancer survivors. Recently diagnosed female breast and colorectal cancer patients using β-blockers reported less cancer-related psychological distress [115]. Although direct psychological stress may not be an important feature in canine cancer, propranolol could potentially help to control the anxiety associated with repeated hospital visits required for treatment.

Side effects of propranolol reported in humans most commonly include hypotension, hypoglycemia, bradycardia, bronchospasm, sleep disturbances (nightmares), acrocyanosis (cyanotic extremities). Other fewer reported side effects include gastroesophageal reflux, nausea, vomiting, diarrhea, somnolence, hyperkalemia, tumor lysis syndrome, psoriatic drug rash, respiratory syncytial virus exacerbation, and dental caries [116-119]. Propranolol is contraindicated in patients with asthma, heart block and sinus bradycardia. The most serious side effect of propranolol is hypoglycemia [120, 121]. Propranolol is thought to cause hypoglycemia by inhibiting glycogenolysis, glyconeogenesis, and lipolysis. Children have lower glycogen stores and higher glucose consumption rates when fasting and, therefore, are more susceptible to
hypoglycemia than adults [122]. Side effect incidence, however, does not appear high. A recent retrospective study revealed that only 7.6% (10/132) of the children who received propranolol treatment had to discontinue it due to side effects. No adverse effects requiring hospitalization were recorded. [123].

In dogs, propranolol is indicated for the treatment of hypertension, atrial fibrillation, tachyarrhythmias [124, 125], myocarditis [126], ventricular premature contractions and arrhythmias caused by digitalis toxicity [127]. It is also useful in the treatment of hypertrophic cardiomyopathy especially when associated with hyperthyroid disease in cats (as it inhibits conversion of thyroxine to triiodothyronine) [128] and for urinary retention (stimulates bladder contraction) [129]. It is contraindicated in patients with congestive heart failure unless it is secondary to a tachyarrhythmia responsive to beta-blockade. The drug is also contraindicated in patients with 2nd or 3rd degree heart block, sinus bradycardia, asthma and thromboembolic disease. It should be used with caution in diabetic patients due to risk of hypoglycemia as described in humans. Side effects are similar to humans and include hypotension, bradycardia, hypoglycemia, decreased cardiac contractility, bronchoconstriction, peripheral vasoconstriction and diarrhea. It is recommended that propranolol therapy be gradually withdrawn due to possible “rebound” effect of catecholamines to the chronically suppressed β2-AR, leading to tachycardia, arrhythmias and hypertension.

Possible drug interactions with propranolol include [130]:

- Delayed gastrointestinal absorption of propranolol by antacids
- Additive toxic effects when used concomitantly with quinidine, procainamide and lidocaine (although anti-arrhythmic effects are enhanced)
• Hypotensive effects are enhanced by chlorpromazine, cimetidine, furosemide, phenothiazines and hydralazine
• Increased serum levels of lidocaine
• Increased effects of tubocurarine and succinylcholine
• Increased action of terbutaline
• Antagonizing effects on epinephrine and phenylpropanolamine
• Antagonizing bronchodilatory effects of theophylline
• Bradycardia may be potentiated by concurrent use of digitalis
• Anti-hypertensive effects of propranolol can be inhibited by concurrent use of salicylates
• Propranolol’s effects can be decreased by concurrent use of thyroid hormone supplementation and the dose of propranolol may require a decrease in animals receiving methimazole

A pharmacokinetic study of propranolol in dogs used a racemic mixture as prepared for pharmaceutical use. A half-life of 1.09 +/- 0.33 hours was observed, which was not different than what was previously reported for a levo isomer given as an IV bolus, however the distribution volumes were significantly greater. A mean value of 6.5 L/Kg was observed with a range from 3.44 to 10.47 L/Kg. Total body clearance was calculated to average 68 mL/min/Kg. Both hepatic and extra-hepatic clearance of propranolol was suggested, and approximately 90% of propranolol is extracted from the blood as it passes through the liver. This extensive extraction results in poor bioavailability, with only 2-17% of the dose reaching the systemic circulation unchanged. Systemic availability increased substantially with multiple dosing (2.1-5.5 mg/Kg every 6 hours), reaching up to 10.7 times greater than predicted by single dose. Propranolol is rapidly absorbed
with an absorption half-life of 17 minutes. Mean CpMax measured in 5 dogs receiving 40 mg (2.1-5.5 mg/Kg) in multiple oral doses was 79.54 ng/mL (range 21-162.5), which is equivalent to a mean of 0.3 µM (range 0.08-0.62 µM) [127]. Another recent study with single administration of three 40 mg tablets revealed a Cmax of 191.4 +/- 56.52 ng/mL (0.73 +/- 0.21 µM) [131].

Propranolol is available in tablets containing 10, 20, 40, 60, 80, 90 mg and oral solution containing 4 and 8 mg/m. Injectable propranolol is available in 1 mg/mL concentration. Standard dosage in dogs: 0.1-0.2 mg/Kg orally every 8 hours (maximum 1.5 mg/Kg), intravenously 0.02 mg/Kg over 5-10 minutes (maximum 1 mg/Kg) [132, 133].

2.5. **Propranolol and infantile hemangioma**

Fairly recently (2008), propranolol was serendipitously discovered to be effective for the treatment of infantile hemangioma after two children who were treated with propranolol for cardiomyopathy were observed to have their hemangioma regress [5]. Based on this observation, the authors decided to treat nine additional children with propranolol and observed similar results. Prior to this discovery, hemangiomas were treated with corticosteroids as a first line of therapy, with other options including interferon alpha, vincristine or surgical removal if the hemangiomas were progressing despite high dose of corticosteroids [134, 135]. Propranolol thus came as an effective, inexpensive option for treatment of complicated hemangiomas.

Infantile hemangiomas (IH) are the most common tumors of early childhood, affecting about 5-10% of infants. It is three times more common in females than males, and most prevalent in Caucasian children [136]. In general is not considered a severe disease, since most IH will spontaneously regress necessitating no additional treatment. However, approximately 12% of IH
are complicated cases in which the location or the speed of growth can promote significant morbidity including disfigurement, ulceration, bleeding, visual compromise, airway obstruction, congestive heart failure and rarely death [137]. Most IH undergo rapid proliferation during the first months to year of life, reaching an average size of 2-20 cm. These lesions then undergo a slow involution period over several years and are generally fully regressed by 5-10 years of age however the duration and rate of growth are variable [136-138].

Histologically these tumors are composed of a mixture of clonal endothelial cells associated with pericytes, dendritic cells and mast cells [139]. They are densely packed over-proliferating capillaries with the absence of an open lumen. The origin is still unclear with some studies suggesting aberrant transplantation of placental endothelial cells [140], predisposing genetic factors [141, 142] and/or tumor stem cell components [136, 143].

The molecular mechanisms behind the development of infantile hemangioma are still under investigation. In the early phase of the disease development, angiogenic and growth factors can contribute for tumor progression; in the late and involuting phase of the disease it is thought that apoptosis is involved. The proposed mechanisms of propranolol effects include inhibition of angiogenesis; early vasoconstriction of the tumor (attributed to the blocking of nitric oxide production) which causes the softening of the tumor as well as change in color from red to purple and later promotion of tumor apoptosis [5].

During the growth or proliferative phase, two main pro-angiogenic cytokines have been implicated: VEGF and bFGF [139, 144-146]. Studies have shown that both endothelial and interstitial cells are actively dividing in that phase. Propranolol has been implicated in suppression of VEGF protein expression in hemangioma-derived endothelial cells in a dose-
dependent manner (25-100 µM) and induced apoptosis by activation of the caspase cascade (caspase 3 and caspase 9) after 100 µM treatment of propranolol [147]. Serial serum VEGF decrease was also demonstrated in clinical patients after initiating propranolol treatment. This same study demonstrated a decrease in serum MMP-9 as well, suggesting another mechanism of tumor control by propranolol [148]. Another study demonstrated a decrease in the VEGFR (VEGF receptor) protein expression after 48 and 96 hours of cell treatment with higher concentrations of propranolol (200-300 µM). Interestingly, there was upregulation of mRNA expression but downregulation of VEGF by propranolol, indicating an inhibition in mRNA translation into VEGF protein. This inhibition in VEGF was mediated by hypoxia-inducible factor 1-alpha (HIF-1α), which is a transcription factor that becomes stabilized in hypoxic situations. In a normoxic environment, HIF-1α is rapidly degraded; in hypoxia it persists as a transcription factor for many genes including VEGF, ultimately contributing to angiogenesis. Another finding was decrease in phosphorylation of PI3/Akt and p38/MAPK in a dose-dependent fashion [149]. In another study, there was an increase in phosphorylation of p38 in propranolol-treated IH. Since p38 regulates the production of inflammatory mediators such as TNFα, IL1β and COX-2 [150], one proposed mechanism of IH remission could be due to immune-mediated responses [136]. Propranolol treatment also caused decreased levels of phosphorylated cofilin. Cofilin is a cytoskeletal-binding protein critical for actin microfilament dynamics and reorganization that severs and depolymerizes actin filaments [151]. Cofilin phosphorylation could lead to increased cofilin-mediated actin severing, which would disrupt cell migration and affect cell proliferation because the actin cytoskeleton is intimately related to regulation of the cell cycle progression [136, 152]. Propranolol also disrupted the cell cycle by decreasing the expression of key cyclin proteins (cyclin A1, A2, B2, D2 and D3) and increased
the expression of important cell cycle inhibitors (p15, p21, p27). No change was noted on expression of cyclin-dependent kinases Cdk2 and Cdk4 [136]. Propranolol inhibited cell proliferation at an IC50 of 50 µM and induced an increase in the proportion of cells in the G1 phase while reducing the proportion of cells in S and G2/M phase [136]. Propranolol did not induce apoptosis of endothelial cells at 50 µM, nor promoted cleavage of caspase 3 and caspase 9, in contrast with pro-apoptotic effects stated by other studies described in this text. Cell migration (assessed by the scratch/wound healing assay) was significantly inhibited after endothelial cells were treated with propranolol at 50 µM for 12 hours [136].

HIF-1α serum levels were found elevated in children with proliferative hemangiomas [153, 154]. HIF-1α protein was decreased in a dose-dependent manner after treatment with propranolol, which was evaluated both by Western blot and ELISA. Another possible pro-angiogenic pathway, NF-κB (nuclear factor kappa-β), was analyzed and was upregulated by treatment with propranolol, indicating a possible compensatory angiogenic mechanism by the tumor [149]. NF-κB constitutes a non- HIF-1α dependent pathway resulting in VEGF-A expression and angiogenesis [155]. Interestingly, the same experiments looking at cell viability, migration, tubule formation, RT-PCR, flow cytometry, western blots and ELISA were performed in hemangioma endothelial cells and repeated in human umbilical vein endothelial cells (HUVEC) used as control cells, with the downregulation of VEGF, VEGFR and HIF-1α not observed in control cells, indicating that the effects of propranolol were confined to the hemangioma endothelial cells only. Propranolol was also observed to inhibit cell migration and tubule formation. Apoptosis of hemangioma endothelial cells, but not hemangioma stem cells, was demonstrated in another study [149]. This could explain the rebound growth of hemangiomas after propranolol therapy has been discontinued [156-158]. Propranolol also
promoted adipogenic differentiation of hemangioma stem cells (involution) [159]. In another study propranolol did not promote apoptosis of hemangioma stem cells at a concentration of 50 µM; the half maximal inhibition was 133 µM, which is well above the biologically achievable dose in humans. Inhibition of proliferation was also not achieved in this study. High levels of VEGF and bFGF were observed in proliferating IH tissue. At low concentrations (0-20 µM), propranolol was capable of decreasing the VEGF mRNA and VEGF protein levels in hemangioma-derived stem cells (HemSCs). There was downregulation of bFGF by HemSCs however it was less pronounced than VEGF. The proportion of VEGF-positive HemSCs was very low in IH tissue [160]. Zou et al showed that propranolol did not affect proliferation of endothelial progenitor cells, but it did inhibit migration of these cells in a dose-dependent (0-100 µM) and time-dependent (24-72 hr) manner. This same study investigated the effects of propranolol in the expression of CXCR4, which was suppressed via Akt and MAPK pathways [161]. CXCR4 is a receptor for stromal cell derived factor 1α, which promotes mobilization of the endothelial progenitor cells from the bone marrow to the site of vasculogenesis [154].

In summary, while there is still active research into the molecular pathways involved in the response of infantile hemangioma to propranolol, most studies suggest that the effects of propranolol are related to suppression of angiogenesis. Some studies also indicate an effect in endothelial cell proliferation and survival.

2.6. **Canine Hemangiosarcoma**

Hemangiosarcoma (HSA) is a malignant neoplasm of vascular endothelial origin. This cancer occurs more frequently in dogs than in any other species [2, 3]. It accounts for 2.3 to 3.6% of skin tumors in dogs and 45 to 51% of splenic malignancies [2, 3, 162-164]. Like majority of
cancers, it affects mostly middle-aged to older animals [163, 165-168]. Breeds that appear overrepresented include German shepherds, Golden retrievers and Labrador retrievers [163, 165, 168-170]. The most common primary site for HSA is the spleen, with other frequent sites include the right atrium, skin, subcutis and liver [1, 2, 165, 166, 168, 169, 171-174].

Being a vascular malignancy, molecular pathways involving angiogenesis have been explored to further clarify the development of this disease. VEGF expression has been demonstrated in canine studies [175-177] and plasma levels of VEGF were higher in dogs with HSA compared to healthy dogs [178]. There was no marked difference between VEGF levels in effusions associated with malignant versus nonmalignant diseases [179]. Other angiogenic cytokines important in HSA include bFGF and angiopoietin 1 (Ang-1) [177, 180]. These cytokines can be secreted by the tumor cells leading to an autocrine growth signaling or can be secreted by other cells within the microenviroment leading to paracrine stimulation. Another study showed a significantly higher proportion of platelet derived growth factor (PDGFR-β) expression in HSAs compared to cutaneous hemangiomas [181].

Mutations in tumor suppressor genes could provide a possible etiology for the disease, however studies suggested that p53 (tumor suppressor gene) and Ras (oncogene) mutations are infrequent in canine HSA [182-184]. PTEN (tumor suppressor gene) inactivation was demonstrated in greater than 50% of evaluated canine HSA samples in one study [185]. Key growth and apoptosis regulating proteins such as pRB, cyclin D1, Bcl-2 and survivin appear overexpressed in HSA when compared with hemangiomas or normal tissues [183, 186].

Histopathology is often necessary to establish a diagnosis, since cytology is non-diagnostic in the majority of time due to excessive bleeding and lack of exfoliation by the tumor
Histologically HSA consists of immature, pleomorphic endothelial cells forming vascular spaces containing variable amounts of blood and/or thrombi [188]. Immunohistochemistry for factor VIII (Von Willebrand) or CD31 can be used to confirm endothelial origin and support the diagnosis [188]. Claudin-5 and CD117 have also been identified as potentially useful markers. (Kit) [176, 189].

Surgery is the main method of treatment for HSA. Adjuvant chemotherapy is indicated in most cases (except for dermal hemangiosarcoma) [190]. Protocols involving doxorubicin are most commonly used, including VAC (vincristine, doxorubicin and cyclophosphamide) [191, 192]; vincristine, cyclophosphamide and methotrexate [168]; doxorubicin and cyclophosphamide; doxorubicin and minocycline [193] and single-agent doxorubicin [194-197]. Ifosfamide has also been used [198, 199]. Epirubicin and intracavitary pegylated liposomal encapsulated doxorubicin do not appear to have advantage over conventional doxorubicin [200, 201]. Immunotherapy has been investigated as possible treatment strategy, with one study using a mixed killed bacterial vaccine following surgery showing some improvement in survival time [168], and another study looking at doxorubicin/cyclophosphamide combination with liposome-encapsulated muramyl-tripeptide-phosphatidylethanolamine (L-MTP-PE) which showed a significant increase in median survival time (5.7 to 9 months) with 40% of the dogs experiencing long term survival [202]. Radiation therapy is mainly used for non-visceral hemangiosarcoma and although it can cause reduction in tumor size it does not significantly change the overall survival [203]. A small study looking at a metronomic chemotherapy protocol using NSAID, cyclophosphamide and etoposide showed a similar outcome to doxorubicin-based protocols [204]. A combination of a dose-intensified doxorubicin protocol with deracoxib (COX2 inhibitor) was well tolerated but did not result in overall improvement of survival in dogs with
splenic HSA [205]. A study investigating the effects of toceranib phosphate (Palladia®), a multiple kinase inhibitor (including VEGFR), administered after splenectomy and 5 doses of doxorubicin given at 2-week intervals has been conducted and the results showed that the administration of toceranib did not significantly improve disease-free interval or overall survival time [206].

The prognosis for canine HSA is very poor. Hemangiosarcoma typically has a very aggressive biologic behavior, with disseminated metastasis occurring very early in the development of the disease, except for dermal hemangiosarcomas. Metastasis is typically hematogenous or through transabdominal implantation following rupture. The most frequent metastatic sites are the liver, omentum, mesentery and lungs. Other reported metastatic sites include kidney, muscle, peritoneum, lymph nodes, adrenal gland, brain and diaphragm. Dogs treated with splenectomy alone have a survival time that range from 19-86 days and less than 10% of dogs living one year [3, 164, 168, 207, 208]. Surgery plus adjuvant chemotherapy will increase median survival times to 141-179 days, however the one year survival rate is still less than 10% [191, 194, 195, 198, 200]. The average survival time of right atrial HSA undergoing surgery is 1-4 months [209, 210]. Dermal HSA treated with surgery alone had a median overall survival time of 987 days and lingual HSA had a median overall survival time of 553 days [211, 212]. One analysis of intramuscular and subcutaneous HSA identified 71 cases, the median time to tumor progression and overall survival time were 116 and 172 days, respectively and 25% survived to 1 year [213]. In summary, the literature on canine HSA describes an aggressive cancer. Unfortunately, there has not been substantial improvement in the outcome of dogs with HSA and therefore novel therapies are needed.
Investigations into the beta adrenergic presence in canine hemangiosarcoma as well as the effects of its blockade on cell activity are starting to emerge. One study that evaluated canine hemangiosarcoma cell lines as well as mouse angiosarcoma, hemangioendothelioma and human dermal microvascular endothelial cell lines revealed that propranolol selectively inhibited proliferation, survival, and migration of a panel of malignant vascular tumor cells, indicating that the oncogenic properties of these tumor types are driven, in part, by beta adrenergic signaling. Propranolol dramatically slowed the proliferation rate of all vascular tumor lines tested, and four of the five tumor lines exhibited nearly 100% lethality at doses that had been previously reported to be non-toxic for primary cultures of human endothelial cells. This finding suggested that malignant endothelial tumors may be more sensitive to beta blockade [214].
CHAPTER 3

MATERIALS AND METHODS

3.1. Cells and reagents

Two cHSA cell lines (DEN and Fitz) were provided by Dr. Douglas Thamm (Colorado State University). Additional cell lines used as controls included HeLa (human cervical adenocarcinoma), MDA-MB-231 (human metastatic breast carcinoma), and MDCK (Madin-Darby canine kidney) purchased from American Type Culture Collection (ATCC). Cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine (2 mmol/L), penicillin (100 IU/ml), streptomycin (100 IU/ml), and 10% fetal bovine serum (FBS) in a humidified atmosphere supplemented with 5% CO₂. Cell cultures were maintained in subconfluent monolayers and passaged as deemed necessary by growth of cell line.

Propranolol hydrochloride (P) was purchased from Sigma-Aldrich (St. Louis, MO - catalog # P0884). Norepinephrine bitartrate salt monohydrate was purchased from Sigma-Aldrich (St. Louis, MO - catalog # A9512). Both reagents were diluted with deionized water based on manufacturer’s instructions and solubility provided.

The anti-β2-AR antibodies (rabbit polyclonal; catalog #AB36956 and #AB135641) used for Western blot (WB) and immunohistochemistry (IHC) experiments, respectively, were purchased from Abcam Inc (Cambridge, MA). The anti-Src (mouse monoclonal; catalog #2110) and anti-phospho-Src (Tyr 416) (rabbit monoclonal; catalog #2113 and rabbit monoclonal; catalog #6943) were purchased from Cell Signaling Technology (Beverly, MA). Antibody anti-tyrosine hydroxylase (rabbit polyclonal, Abcam #AB112) and anti-CD31 (mouse monoclonal,
Dako, North America Inc., #M0823) were used in splenic cHSA tissue samples. The anti-β actin antibody used for loading control (rabbit polyclonal; catalog #AB8227) was purchased from Abcam Inc. Horseradish peroxidase (HRP) conjugated anti-mouse and anti-rabbit secondary antibodies used for WB were purchased from GE Healthcare, UK. Blocking peptide for the β2-AR WB (catalog #38102) was purchased from Abcam Inc.

Other reagents used include WB developing solution (SuperSignal West Femto Chemiluminescent Substrate, Thermo Scientific, Rockford, IL), bovine serum albumin (BSA), milk, tris-buffered saline and Tween 20 (TBST) and hydrogen peroxide (H₂O₂).

3.2. Immunohistochemistry

3.2.1 Spontaneous canine HSA samples

Eighteen canine splenic HSA tissue blocks were obtained from the Veterinary Diagnostic Laboratory of the University of Illinois at Urbana-Champaign. Paraffin-embedded cell pellets were sectioned every 3 microns, placed on positively charged slides, and dried for one hour at 60°C. Slides were deparaffinized with two sequential xylene washes, and subsequently hydrated using two ethanol baths for five minutes each; followed by a water rinse. Slides were placed in 3% H₂O₂ for 15 minutes and rinsed with water. Antigen retrieval was performed with citrate buffer microwaved for five minutes. Nonspecific staining was minimized using a blocking solution for 30 minutes (5% BSA in PBST). Slides were then incubated with rabbit polyclonal anti-β2-AR antibody at a concentration of 1:35 for 30 minutes at 37°C followed by one hour at room temperature. Slides were rinsed twice with PBST and then incubated with a biotinylated secondary anti-rabbit antibody for 30 minutes at room temperature. Slides were washed in PBST
twice before incubation for 30 minutes with a streptavidin–biotinylated HRP complex, and
developed with 3,3-diaminobenzidine (DAB) (BioGenex, Freemont, CA). Slides were then
counterstained with Mayer’s hematoxylin. The negative control samples underwent the same
process but in the absence of the primary antibody. The isotypic negative control was performed
using tyrosine hydroxylase antibody (1:200), which is very specific for neuronal tissue. This was
performed to rule out non-specific Fc receptor binding or other cellular protein interactions, as
well as confirm the specificity of the primary antibody. The rabbit polyclonal anti-β2-AR
antibody was validated using canine heart tissue as a positive control as well as mouse spleen
and kidney [215-217]. All cHSA tissue samples were reviewed by a single pathologist (S.
Lezmi) and the diagnosis of hemangiosarcoma was confirmed with H&E staining and with IHC
using a CD31 antibody. CD31 antigen is a marker for endothelial cells and is expressed only by
endothelial cells, platelets and macrophages; it is conserved in neoplasia arising from endothelial
cells and validated in canine tissue [218]. The intensity of the β2-AR staining was evaluated
(scored as strong, mild or none) as well as the localization within the cell (nuclear vs.
cytoplasmic).

3.2.2. Cell pellets

Fitz and DEN cell lines and a human positive control cell line, MDA-MB-231 [219],
were used to evaluate β2-AR expression using IHC. Adherent cell cultures were collected and
washed in PBS, then pelleted by centrifugation (10,000 RPM for 10 minutes). Each pellet was
re-suspended in 1 ml 10% formalin for one hour. Formalin was removed and cell pellets were
re-suspended uniformly into 1 ml 4% melted agarose gel by vortexing, then immediately
centrifuged to create an agarose-embedded cell pellet. Cell pellets were then trimmed and
processed as for formalin-fixed paraffin-embedded tissue biopsy specimens. Briefly, paraffin-embedded cell pellets were sectioned at three microns, placed on positively charged slides, and dried for one hour at 60°C. Slides were deparaffinized with three sequential xylene washes, and subsequently hydrated using 100%, 95%, and 70% ethanol for two minutes each; followed by a water rinse. Slides were placed in 3% H₂O₂ in methanol for 15 minutes, and then treated with citrate buffer (pH 6.0), placed in a decloaking chamber until the temperature reached 125 °C for 30 seconds with psi between 18-22, followed by 90°C for 10 seconds. Slides were removed from decloaking chamber and cooled for two minutes followed by a wash in Super Sensitive Wash Buffer (BioGenex, San Ramon, CA). The rabbit polyclonal anti- β2-AR antibody was used at a concentration of 1:100 for one hour at room temperature. Slides were rinsed with SuperSensitive Wash Buffer and treated with Super Enhancer (BioGenex, San Ramon, CA) for 20 minutes at room temperature, and subsequently treated with Polymer-HRP (BioGenex, Fremont, CA) for 30 minutes at room temperature and incubated with DAB (BioGenex, Fremont, CA) at room temperature for five minutes. Slides were washed with SuperSensitive Wash Buffer and counterstained with Mayer’s hematoxylin for one minute. Negative control samples underwent the same process but in the absence of the primary antibody.

3.3. Cell protein extraction

Cells were grown in culture until confluence and then media was removed and cells were washed twice with ice-cold PBS. Cells were then placed in trypsin for five minutes to detach from culture plate, followed by addition of complete media to neutralize the effects of trypsin. Non-adherent cells were centrifuged at 2,000 rpm at 4°C for five minutes. Supernatant was removed and the pellet was washed twice in PBS until all trypsin and media was removed. The
pellet was then re-suspended in a solution containing Mammalian Protein Extraction Reagent (M-PER, Pierce, Rockford, IL) and Pierce protease inhibitor cocktail solution (Pierce, Rockford, IL) diluted 1:100 for final working solution, 10 μL of protease inhibitor to 1000 μL of M-PER. This was placed on a shaker for 10 minutes, followed by centrifugation at 10,000 RPM, in 4°C for 10 minutes. The supernatant was placed in separate Eppendorf tubes and placed in -80°C until analysis.

3.4. Western blot analysis

Cellular protein concentrations were determined using a standard assay kit (Bicinchoninic Acid Protein Assay, Rockford, IL). For each protein expression analysis, 50 μg samples were electrophoresed on 12% polyacrylamide gel and then transferred to nitrocellulose membrane. The membranes were blocked with either TBST with 5% milk (β2-AR and Src) or TBST with 5% BSA (p-Src), followed by addition of their primary antibody at their specific concentrations and exposure time as described in the next sections. In all WBs, secondary HRP-conjugated antibody was added at a concentration of 1:1000 for one hour at room temperature after the membranes were washed three times with TBST for five minutes, and developed using chemiluminescent substrate followed by detection with enhanced chemiluminescence (ECL) detection system (Amersham). The blots were imaged using ChemiDoc XRS+ molecular imager system (Bio-Rad Laboratories, Hercules, CA) with Image Lab software (version 5.1 build 8, Bio-Rad Laboratories). The evaluation of protein loading within the blots was performed by incubating the membranes with anti-β-actin antibody (1:2000) without the need for stripping the membranes since the expected molecular weight of β-actin band was below (46 kDa) the other bands evaluated. The membranes were subsequently incubated with the secondary antibody and
imaged as described previously. The β-actin loading control is important to verify that the amount of protein loaded into the gel is approximately equal.

3.4.1 β2-AR

Protein expression of β2-AR was performed via Western blotting using cell lysates from Fitz and DEN, as well as HeLa and MDA-MB-231 cells as positive controls [219, 220]. The primary antibody was diluted in 5% milk in TBST at a concentration of 1:250. Band specificity was confirmed with a matching blocking peptide. For evaluation of the blocking peptide, one membrane containing duplicated samples was separated into two membranes becoming two exact copies. One portion of the membrane was treated first with the blocking peptide (1:250) diluted in 5% milk/TBST for one hour at room temperature, rinsed three times with TBST and then incubated with the primary antibody for one hour at room temperature. The other portion of the membrane was incubated with only the primary antibody (1:250) for one hour at room temperature. Subsequent incubation with secondary antibody and analysis was performed as described above.

3.4.2 Src

Protein expression of Src was performed via Western blotting using cell lysates from Fitz and DEN, and MDCK as a positive control (per antibody manufacturer). Primary antibody (1:1000) was diluted in TBST with 5% milk incubated overnight. Basal Src protein expression was determined in all three cell lines. In addition, protein expression was assessed in Fitz and DEN exposed to different concentrations of NE and P (0.1 and 10 µM) for 24 hours.

3.4.3 Phospho-Src
Protein expression of phosphorylated Src (p-Src) was performed via Western blotting using cell lysates from Fitz and DEN. The membrane was incubated overnight with the anti-phospho-Src (1:1000) antibody diluted in TBST with 5% BSA. For the analysis of phosphorylation status of Src after cell treatment, several experiments were performed under varying experimental conditions. For positive control of Src phosphorylation in all experiments, cells were exposed to 3 mM of H₂O₂ for five minutes per manufacturer’s instructions. First experiment: DEN and Fitz cells were serum-starved overnight then treated with NE (10 µM) or P (0.1 µM) for different time exposures (5-30 minutes). Second experiment: To optimize visualization of the agonism, Fitz cells were serum-starved for 24 hours then treated with NE (10 µM) at increasing time-exposure (5-60 minutes). The goal was to determine if phosphorylation was positive upon contact with the agonist, if that difference was visible in the WB, and how much exposure was necessary for optimal visualization. Third experiment: To determine if propranolol could block NE agonism, Fitz cells were serum-starved for 24 hours, then pre-treated with propranolol (0-0.1 µM) followed by NE treatment (10 µM) at increased time exposures (5-60 minutes). Fourth experiment: To determine if propranolol could rescue NE agonism, DEN and Fitz cells were serum-starved for 24 hours, then pre-treated with NE (0-10 µM) for 45 minutes followed by P (0-100 µM) for 24 hours. Band volume analysis was performed using Image Lab software and results of the Phospho-Src proteins were adjusted for β-actin expression levels used as loading control. For the third experiment, results are expressed as p-Src/ β-actin ratio.

3.5 VEGF ELISA
Fitz and DEN cells were plated at a density of 5 x 10^3 cells/well in a 96-well plate. After allowing cells to adhere to the plate overnight, the medium was removed and replaced with fresh medium containing NE and/or P. In the first experiment, optimal agonism was assessed using 0-10 µM NE for 24 hours. In the second experiment, cells were treated with 0-100 µM P with NE agonism at 1 µM which was selected as the optimal agonist. Cell culture supernatants were harvested and soluble VEGF was determined with a commercially available canine VEGF ELISA immunoassay (Canine VEGF DuoSet, R&D Systems, Minneapolis, MN). Differences in soluble VEGF secreted by the cHSA cells after each treatment were normalized to cell count with the use of a colorimetric proliferation assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay – MTS, Promega, Madison, WI) in which optical density linearly correlates with viable cell numbers. Specifically, normalized VEGF concentrations were based on the average of quadruplicate samples for each experimental group expressed as the following ratio: Normalized VEGF = [Calculated VEGF (pg/mL)]/optical density. Samples were performed with six replicates per experiment and the results were replicated in three independent experiments.

3.6. Proliferation assay

Fitz and DEN cell proliferation was assessed using a commercial colorimetric assay per manufacturer’s directions (CellTiter 96® AQueous One Solution Cell Proliferation Assay – MTS, Promega, Madison, WI). This assay measures the number of viable cells by using a tetrazolium compound that is bioreduced by metabolically active cells into a colored formazan product, which is detected via spectrophotometry. Fitz and DEN were seeded into a 96-well plate at a density of 5 x 10^3 cells/well. After allowing adherence overnight, the cells were treated with 0, 0.1, 1 and 10 µM of NE or P for 24 hours (first experiment) and with 0, 0.1 or 10 µM NE, 0.1
or 10 µM P, 0.1 or 10 µM NE + P (second experiment) for 48 hours. The supernatant was removed, and 120 µL of fresh media containing 20 µL of the reagent was added to each well. The plate was then incubated at 37°C in 5% CO2 for 1, 2 and 3 hours. At each hour, spectrophotometer readings were obtained with a photometric plate reader (Bio-Tek EL 800) at a 490nm absorbance (wavelength correction set to 570 nm). The data collected for treated cells was compared to untreated cells of the same experiment and the difference was calculated as a percentage of control cells. The groups were subsequently compared to each other. Samples were performed with six replicates per experiment and the results were replicated in three independent experiments.

3.7. **Scratch assay**

Analysis of cell migration was done using the “scratch assay” method [221]. Fitz and DEN were grown in culture in 6 well plates until reaching confluence. A scratch was then made along the center of the cell culture area using a p200 pipette tip. The medium was then replaced with fresh medium containing P (0.01-100 µM) or no treatment (control). An image of the scratched cell monolayer was taken (“time 0”) using an inverted microscope (Nikon Eclipse TS100) with a mounted digital camera (SPOT Insight QE model #4.2, SPOT Imaging Solutions, Michigan, USA). Cells were then grown in culture for 24 hours and another image was taken at the same location of the initial image. Captured images were analyzed with Image J software (National Institutes of Health, Bethesda, MD). A total of 5 measurements of the gap between the cells were taken for each image and average measurements were compared between time 0 and 24h. Experiment was performed in triplicate with replication.

3.8. **Statistical analysis**
Data from proliferation, VEGF assay and scratch assay were analyzed for normality with the Kolmogorov-Smirnov test. Because of the small number of samples, histograms were also performed for visual assessment. Data is reported as mean and standard deviation. One-way ANOVA was used to evaluate for difference between groups, with Tukey’s multiple comparison test to detect a difference between groups. GraphPad Instat 3.1 and GraphPad Prism 6.1 software were used for analysis.
CHAPTER 4

RESULTS

4.1. β2-AR is expressed in spontaneous canine splenic hemangiosarcoma

To determine β2-AR protein expression in spontaneous canine hemangiosarcoma, IHC was performed. First, the 18 selected tissue samples were confirmed cHSA based on H&E staining and positivity for CD31. All tissue samples demonstrated positive β2-AR expression (Fig 4.1). Expression of the receptor was identified in the nucleus and cytoplasm, with varying stain intensity between tissue samples. Five of 18 (27%) samples had strong intensity and were almost diffusely labeled. Eight of 18 (44%) of the samples had only cytoplasmic staining while 10/18 (56%) had staining of both the nucleus and the cytoplasm. The labeling was frequently heterogeneous with some cases presenting large unlabeled areas. Isotype control did not reveal any significant non-specific background staining.

4.2. β2-AR is expressed in canine hemangiosarcoma cell lines

To determine β2-AR protein expression in canine hemangiosarcoma cell lines Fitz and DEN, WB and IHC were performed. Using WB, we identified a band at the expected molecular weight of 55 kDa in both human positive control cell lines MDA-MB-231 and HeLa, as well as the canine cell lines Fitz and DEN (Fig. 4.2b). Qualitatively, Fitz appears to have stronger protein expression than DEN. Specificity was confirmed with abrogation of the band in the presence of a blocking peptide. β2-AR protein expression was also confirmed via IHC on Fitz and DEN cell pellets using the same antibody. Cell pellets demonstrated strong, uniform, diffuse staining (Fig. 4.2a).
4.3. Investigating intermediate signaling via Src/p-Src

4.3.1. Src and p-Src are expressed in cHSA cell lines

Src and phosphorylated Src (Tyr416) are intermediaries involved in signal transduction from β2-AR to the nucleus [19]. To determine their involvement in cHSA cells we performed WB. We identified bands (Src and p-Src) at the expected molecular weight of 60 kDa in both canine positive control cell line MDCK as well as the canine cell lines Fitz and DEN (Fig. 4.3a).

4.3.2. Propranolol attenuates norepinephrine-induced Src phosphorylation

Given that Src and phosphorylated Src (Tyr416) are intermediaries involved in signal transduction from the β2-AR to the nucleus, we sought to determine if the β2-AR agonist (NE) would lead to phosphorylation of the protein, and if this process could be blocked by the antagonist (P). To determine the effects of NE and P on Src and p-Src, we performed WB on cHSA cells. In DEN and Fitz cells treated with different concentrations of NE and P (up to 10 μM), there was no qualitative difference in the expression of total Src (Fig. 4.3b). Both DEN and Fitz demonstrated significant basal phosphorylation of Src, suspected to be caused by the FBS used in culture. Overnight serum-starvation in DEN and Fitz cell lines did not result in an apparent difference in p-Src protein expression with P (low dose 0.1 µM) and increased time-exposure to NE (Fig. 4.3c). To optimize phosphorylation, Fitz cells were serum-starved for 24 hours, followed by increasing time exposure to NE. Increased protein expression of p-Src was identified, with phosphorylation more pronounced starting at 15 minutes and persisting through 60 minutes (Fig. 4.3d). Once the positive effects of the agonist NE on phosphorylation were demonstrated, we proceeded to evaluate if pre-exposure to low-dose propranolol (0.1 µM) was
sufficient to block the receptor to the effects of the agonist. In Fitz cells, NE caused a modest phosphorylation of Src and treatment with low-dose propranolol for 24 hours prior to exposure led to a modest decrease in phosphorylation. These effects were visible in the WB and therefore we proceeded to quantify these results by normalizing the adjusted volume of the p-Src band to the matching β-actin band, to ensure that the increased intensity of the band observed was not due to increased protein loading (Fig. 4.3e). To determine if propranolol could rescue NE agonism, DEN and Fitz cells were serum-starved, pre-treated with NE (0-10 µM), then propranolol (0-100 µM). In Fitz cells, low doses of propranolol reduce p-Src compared to untreated cells, which is lost at the highest dose in unstimulated cells. In NE-stimulated Fitz cells, propranolol reduces p-Src compared to basal levels at all doses. Conversely, in DEN cells, propranolol increases p-Src in untreated and NE stimulated cells (Fig. 4.3f)

4.4 Propranolol affects VEGF secretion of chHSA in vitro

After demonstrating the presence of the receptor as well as a possible intermediate protein involved in signal transduction, we proceeded to investigate the effects of NE and P on cell function activities of chHSA cells. Since propranolol’s ability to inhibit tumor angiogenesis has been largely implicated as a primary anti-neoplastic effect, we sought to evaluate the effects of NE and P on the ability of chHSA cells to secrete VEGF, a key pro-angiogenic cytokine. In the first experiment, we used NE at varying doses (0-10 µM) to assess optimal agonism. Normalized to cell count, basal secretion of VEGF was 1098.83 pg/mL and 464.42 pg/mL for DEN and Fitz cells, respectively. All doses of NE induced VEGF secretion compared to untreated cells for both cell lines. Optimal NE agonism was obtained at 1 µM NE, with a significant increase in VEGF secretion compared to control. At optimal NE agonism, there was an increase in VEGF of 29%
for DEN cells (p<0.05 for all treatments compared to control), and 2.6-fold increase for Fitz cells (p<0.001 for all treatments compared to control) (Fig. 4.4a). Cells were then incubated with the selected NE dose of 1 µM combined with varying doses of P starting at a biologically achievable dose (0.1 µM) up to 100 µM. No significant differences were noted at low doses, however there was a reduction in VEGF secretion in DEN at P ≥ 10 µM (p<0.01) with a maximum decrease in VEGF of 21% at 100 µM. Similar results were seen with Fitz, with reduced VEGF secretion at P ≥ 10 µM (p<0.001) with maximum 44% reduction at 100 µM; a dose dependence was noted in the Fitz cell line (p<0.05) (Fig 4.4b). Similar but less profound results were seen without NE stimulation (results not shown).

4.5. Propranolol affects cell proliferation of cHSA in vitro

The metabolic activity of Fitz and DEN, an indirect measurement of cell viability and proliferation, was evaluated upon exposure to different doses of NE and P. While there was no significant difference noted after 24h incubation, when treated for 48h Fitz exhibited significant difference in proliferation between untreated vs 0.1 µM of NE (25% increase) (p < 0.05) and untreated vs 0.1 µM of P (30% increase) (p < 0.01). DEN did not exhibit significant difference between groups (p > 0.05) (Fig. 4.5)

4.6. Propranolol affects cell migration of cHSA in vitro

The ability of cancer cells to move within the microenvironroment towards the nearby vasculature is important in the process of metastasis and therefore we elected to evaluate if P had
any effect on cell mobility by evaluating the cells ability to close a gap created between the cells. Increasing concentrations of P were used (0-100 µM) and an artificially created gap was measured before and 24 hours after treatment. Data is represented as a percentage of the original gap (time 0), with being 100% a completely closed gap. The gap remained more noticeably open at 10 and 100 µM, being statistically significant at 100 µM when compared to untreated control cells (p<0.05) (Fig. 4.6).
CHAPTER 5

DISCUSSION AND CONCLUSIONS

In veterinary medicine, the emotional component of stress may not play an important role as it does in humans, however the presence of cancer can lead to microenvironmental stressors such as hypoxia and inflammation which may lead to catecholamine release [222, 223]. Therefore, stress can play a part in canine tumor progression. In this study we were able to demonstrate the presence of β2-AR in all spontaneous splenic cHSA as well as cell lines demonstrating the clinical relevance and consistency of expression, and suggesting that this pathway may be activated by stress-related catecholamines. The Fitz cell line appeared to have stronger protein expression than the DEN cell line. The expression of β2-AR in spontaneous tumor samples was heterogeneous in intensity and localization within the cells. The variability of IHC staining could be explained by a variable expression of the receptor within the neoplastic cells, as well as integrity and detectability of the antigen depending on the time the tissue has been stored and the fixative used to initially preserve it as described in a study looking at EGFR [224, 225]. Since the samples tested were from different cases obtained over several years, the intensity of the staining obtained could be a reflection of the particular sample rather than variability within the tumors. However, since tumors undergo frequent mutations, some may express more of a particular antigen or receptor compared to others and this variability may account for the difference between cytoplasmic versus nuclear staining. Regarding cellular localization, upon stimulation β2-AR undergoes internalization, intracellular trafficking and recycling and thus variability in cytoplasmic localization may reflect variation in stimulation and internalization [226]. Nuclear membrane localization of β1- and β3-AR as well as downstream
signaling partners, but not β2-AR, has been reported in normal cardiac myocytes [227]. Our IHC results are in concordance with similar studies in human cancer. In a study of β2-AR IHC expression in hepatocellular carcinoma, 60% of tumors had moderate/strong staining intensity, 31% weak staining intensity and 9% negative staining with nuclear and cytoplasmic distribution [228]. Another study showed strong β2-AR staining in 41% of angiosarcoma samples that were analyzed and a 77% total expression indicating heterogeneity in receptor expression, similar to the results obtained in this project [34]. Further evaluation of localization of β2-AR and signaling partners could include Western blotting of fractionated cell lysates, confocal microscopy with immunofluorescence and other methods.

The non-receptor tyrosine kinase Src has been reported as one of the many downstream proteins involved in β2-AR activation and therefore we elected to investigate if this pathway is indeed activated and blocked by β2-AR agonism/antagonism [19]. After initially demonstrating the presence of Src within the cell, we proceeded to verify if this protein is phosphorylated by NE. The initial challenge was to overcome the strong basal phosphorylation of this protein within the cHSA, which did not allow for a difference in visualization between untreated and treated cells. Part of this phosphorylation was probably due to the presence of FBS, which contains growth factors that can stimulate the Src pathway. After 24h serum-starvation there was still p-Src activity within the untreated cells, however it was diminished enough that an appreciable difference was seen compared to NE-treated cells. Norepinephrine agonism of Src phosphorylation was confirmed, supporting a role for catecholamine-induced tumor stimulation. The subsequent experiment was performed with pre-treatment of cells with P, showing a decrease in phosphorylation at low doses of P. With NE agonism prior to P treatment (i.e. propranolol “rescue”), which may most closely resemble the clinical scenario, P successfully
decreased Src phosphorylation in NE pretreated cells, while phosphorylation was variable in
DEN cells. This may be a factor of β2-AR expression, which was qualitatively higher in Fitz
cells. The preliminary results of this experiment warrants further investigation of this pathway,
however due to multiple pathways involved in β2-AR signaling it is possible that alternative
pathways are stimulated or inhibited with propranolol leading to the effects seen. These effects
were related to proliferation, VEGF secretion and cell migration. With respect to VEGF
secretion, cHSA cells secrete basal levels of VEGF with NE causing increased VEGF secretion,
more evident in the Fitz cell line. This supports our hypothesis that NE would increase VEGF
secretion as demonstrated in other studies [229]. The increase in VEGF may be a result of Src
phosphorylation as hypothesized, and the more profound reduction in VEGF in the Fitz cell line
may be related to the increased β2-AR expression as well as the more consistent phosphorylation
effects of P. Taken together, this data supports a role for the β2-AR and the potential role of
propranolol in inhibiting VEGF-mediated HSA angiogenesis.

The effects of NE and P on cell proliferation were also investigated. The initial
hypothesis was that NE would lead to increased proliferation and P would decrease proliferation
[230]. Interestingly, we found that both NE and P caused an increase in proliferation in Fitz cells
at 0.1 µM. There is one report in the literature of P inhibiting proliferation of pulmonary artery
smooth muscle cells at a 10 µM but not at 0.1 µM. The mechanism for this effect on proliferation
remained unexplained [231]. One possible explanation may be that this β-blocker could act as an
agonist to a different receptor or pathway involved in cell proliferation. Additional studies such
as time-dependent effects may elucidate this further. In one rodent study, propranolol caused a
transient increase in markers of cell proliferation (PCNA, mitotic index) that were abrogated
with longer term treatment [232]. The variation observed between the different cell lines in this
project was also observed in another study, where beta blockade effectively reduced proliferation rate among multiple cell lines however some lines were more resistant to these effects than others [214].

Our last finding was that P at a high dose (100 µM) had an effect on cell migration. This effect has been described previously in human infantile hemangioma endothelial cells and it also decreased phosphorylation of cofilin, as a possible mechanism involved (since cofilin is an actin-severing protein and phosphorylation is an inhibitory event) [136, 233]. The dose necessary for a significant effect is not biologically achievable, however a time-dependent effect at lower doses was not investigated in this study and is an avenue for further investigation. Decrease in cell migration was detected at a 50 µM dose in one study, which was careful to evaluate the effects of this dose on net cell proliferation to eliminate inhibition of proliferation as a contributing factor [214]. It is possible that our dose of 100 µM is affecting cell proliferation, which is indirectly leading to part of the migration effects seen on the scratch assay.

In relating this study to the clinical scenario of canine hemangiosarcoma, the finding of β2-AR expression in both spontaneous tumors and two cell lines, strong basal phosphorylation of Src, as well as consistent agonism of downstream signaling by NE supports a role of this pathway in tumor progression. Variability in expression may suggest a variable response to pathway blockade. Effects on VEGF secretion and cell migration were most significant at the highest doses of propranolol, which are not biologically achievable with single-dose treatment at standard doses. The high doses of propranolol needed to impair tumor cell function is consistent with that seen in infantile hemangioma – most of these studies show *in vitro* effect at 100-300 µM, which is not clinically achievable. However, in infantile hemangioma, continuous treatment is used clinically, which may account for the discordancy between *in vitro* and *in vivo* effect. The
finding of positive effects on Src phosphorylation at low doses in cHSA may also support a potential for low-dose propranolol time-dependent effects on VEGF secretion or cell migration, although this was beyond the scope of this study. Continuous low dose treatment with propranolol would be reasonable in an adjunctive setting in cHSA due to its low cost and side effect profile.

Although this study indicated the presence of the β2-ARs in cHSA and that propranolol exerts some biological effects in cHSA cell lines, it is important to acknowledge some limitations of this project. There is no direct evidence that Src is the intermediary pathway connecting β2-AR agonism and cell proliferation, VEGF secretion or cell migration since there are many other possible pathways involved [19]. We attempted to elicit phosphorylation of Src by exposing the cells to NE over time in order to detect the optimal moment at which most of the protein is phosphorylated and thus more easily detectable. We were only able to detect a small percentage of phosphorylation and even smaller attenuation by propranolol in one experiment. The necessary amount of phosphorylated Src protein required for downstream activation of subsequent proteins (such as STAT3 [234]) is unknown and could be as low as 1% and as high as 100%, therefore the amount of necessary blockade of phosphorylation for biological effect could also be variable. The use of an agonist with a stronger affinity for β2-AR, such as isoproterenol [235], could lead to greater phosphorylation of Src and allow for more objective visualization of the effects of propranolol. The cells were treated with 0.1 µM of propranolol for one hour, which is a biologically achievable dose; however it does not represent the exposure time to which an animal is subjected to, being a drug of continuous use. The same critique can be applied to the other assays in which the cells were treated for either 24 or 48 hours, revealing only statistically significant results at higher doses, which are not biologically achievable. Most
studies looking at the *in vitro* effects of norepinephrine or propranolol used high doses of agonist/antagonist, probably because it was necessary to achieve significant findings. Drug absorption and delivery to the tumor can be variable in each patient depending on tumor blood flow and microenvironment, which is one limitation of translating *in vitro* findings to *in vivo* effects. Additionally, as noted in one study where propranolol did not completely abolish tumor cell proliferation *in vivo* as noted by positive PCNA staining in propranolol-treated tumors, single-agent propranolol may not be as effective in tumor control however synergistic effects may be observed with chemotherapeutics warranting further investigation into combination therapy [214].

In conclusion, our study showed that cHSA express β2-ARs, Src kinase is one pathway activated by this receptor and agonism/antagonism of this receptor exerts anti-angiogenic effects in VEGF secretion and minimal effect on cell proliferation. Propranolol at high doses affects cell migration. Further studies looking at other possible intermediate pathways as well as other mechanisms involved in cancer development (matrix metalloproteinases or apoptosis) are areas for further investigation.
CHAPTER 6

FIGURES

Figure 2.1. G Protein-coupled receptor activation (Belmonte S.L and Blaxall B.C., Circulation Research 109 (3), 309-319, July 2011).

Figure 2.2. G Protein-coupled receptor downstream signaling. (Cole S.W. and Sood A.K., Clin Cancer Res 18 (5), 1201-1206, March 2012).
Figure 4.1. Expression of β2-AR in spontaneous cHSA. Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections containing tissue from splenic cHSA. All samples stained positive for the β2-AR antibody and the degree of staining was variable. Examples of strong β2-AR stain intensity (top row) and mild β2-AR stain intensity (bottom row) are shown. The diagnosis of splenic cHSA was confirmed after review of H&E staining and CD31. Negative control was performed using an isotypic control antibody (1000x).

![Image](image1)

Figure 4.2a. Expression of β2-AR in cHSA cell lines. Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections of agar-embedded cell pellets containing cHSA cells Fitz and DEN. Human mammary carcinoma cell line MDA-MB-231 was used as a positive control. Negative control was the absence of the primary antibody (10x).

![Image](image2)
Figure 4.2b. Expression of β2-AR protein in cHSA cell lines. Western blot was performed using whole cell lysates of Fitz and DEN. Cell lines HeLa and MDA-MB-231 were used as positive control.

![Western blot of β2-AR and β-actin](image)

Figure 4.3a. Expression of Src and p-Src in cHSA cell lines. Western blot using an antibody for Src and p-Src was performed using whole cell lysates of Fitz, DEN and MDCK (control). For better visualization of p-Src the cells were serum-starved and stimulated with 3 mM of H2O2 for 5 minutes. All cells demonstrated the presence of basal Src protein as well as phosphorylated Src protein.

![Western blot of Src and p-Src](image)

Figure 4.3b. Expression of Src in cHSA cell lines exposed to different concentrations of NE and P. Fitz and DEN cells were treated with different concentrations of NE and P for 24 hours as indicated below. Western blot revealed bands of similar size across all treatments, indicating that the presence of total Src protein is independent of the stimulus.

![Western blot of Src](image)
Figure 4.3c. Expression of p-Src in cHSA cell lines during increasing exposure to NE and P. Fitz and DEN cells were serum-starved overnight and treated with 10 μM of NE and 0.1 μM of P for different lengths of time as indicated below. Despite 12hr serum-starvation there was still marked baseline phosphorylation of Src, which did not allow for qualitative distinction between control and treated groups.

Figure 4.3d. Expression of p-Src in Fitz cell line during increasing exposure to NE. Fitz was serum-starved for 24 hr and subsequently increasingly exposed to 10 μM of NE. There was a qualitative difference between untreated control and 15-60 minute exposure.
Figure 4.3e. Expression of p-Src in Fitz cell line during increasing exposure to NE after pretreatment with P. Fitz was serum-starved for 24 hr and cells received either no pre-treatment or 0.1µM of propranolol for 24 hours. Both groups were subsequently increasingly exposed to 10 µM of NE. There was a modest increase in phosphorylation of Src over time (23% at 60’) and there was modestly decreased phosphorylation when the cells had been pretreated with propranolol (11% at 60’). This difference was quantified as the ratio of chemiluminescence emitted by p-Src and β-actin for each band.

![Chemiluminescence graph](image)

Figure 4.3f. Expression of p-Src in DEN and Fitz cell line pretreated with NE and rescued with propranolol. DEN and Fitz were serum-starved for 24 hr and cells received either no pre-treatment or 10µM of NE for 45 minutes. Cells were subsequently exposed to 0-100µM of P. In non-pretreated Fitz cells, low doses of propranolol reduce p-Src, which is lost at the highest dose. With NE agonism in Fitz cells, propranolol reduces p-Src compared to basal levels at all doses. In DEN cells, propranolol increases p-Src in untreated and NE stimulated cells.

![Protein expression graph](image)
Figure 4.4a. VEGF secretion by cHSA cell lines after treatment with NE. The graph represents an average of VEGF secretion by Fitz and DEN quantified by ELISA and normalized to cell count. Cells were treated with increasing doses of NE (0-10 µM) for 24 hours. In both cell lines, compared to untreated cells, there is a significant difference in VEGF secretion (DEN p<0.05, Fitz p<0.001). Error bars represent SD.

![Graph showing VEGF secretion by cHSA cell lines after treatment with NE.](image)

Figure 4.4b. VEGF secretion by cHSA cell lines after treatment with NE and P. The graphs represent an average of VEGF secretion by Fitz and DEN quantified by ELISA and normalized to cell count. All cells were treated with 1 µM NE (agonist) and P at 0-100µM for 24 hours. At propranolol doses of ≥10 µM, there was a significant difference of VEGF secretion by both cell lines compared to untreated cells (DEN p<0.01, Fitz p<0.001). For Fitz, there was a significant difference between 10 and 100 µM, p<0.05. Error bars represent SD.

![Graph showing VEGF secretion by cHSA cell lines after treatment with NE and P.](image)
Figure 4.5. Proliferation activity of cHSA cell lines after treatment with NE and P. Fitz and DEN were treated with different concentrations of NE and P for 24 hours (top two graphs) and subsequently analyzed by MTS assay. There was no statistical difference between the groups in both cell lines (p > 0.5). When cells were treated for 48 hours (bottom 2 graphs), Fitz exhibited significant difference between untreated vs 0.1 µM of NE (p < 0.05) and untreated vs 0.1 µM of P (p < 0.01). Error bars represent SD.
Figure 4.6. Migration of cHSA cells when exposed to increasing doses of propranolol. A scratch assay was performed with Fitz and DEN exposed to increasing concentrations of propranolol for 24 hours, and an average measurement of the residual gap in each treatment group was compared to an untreated control. There was a significant difference between the untreated group and 100 µM (p < 0.05). Error bars represent SD.
Figure 4.6 (cont). Migration of cHSA cells when exposed to increasing doses of propranolol. A scratch assay was performed with Fitz and DEN exposed to increasing concentrations of propranolol for 24 hours, and an average measurement of the residual gap in each treatment group was compared to an untreated control. There was a significant difference between the untreated group and 100 µM (p < 0.05). Error bars represent SD.
REFERENCES


