THE COMPARATIVE EFFECTS OF ACID SUPPRESSANTS ON HEALTHY AND NEOPLASTIC *IN VITRO* MAST CELLS

A Dissertation

by

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ABSTRACT

Mast cell tumors (MCTs) are common skin tumors in dogs and are treated surgically when possible. Because of their integral role as sentinels of immunity, release of inflammatory mediators by MCTs during activation (e.g. degranulation) can have catastrophic consequences. For example, histamine release triggers angioedema, tissue necrosis, gastrointestinal ulceration, and tumor-related death. Prior to surgery, or in the case of non-resectable tumors, adjunctive treatments with drugs that are cytotoxic to mast cells (MCs) or prevent MC release of inflammatory mediators would be of great benefit. We believe that acid suppressant medications might directly kill MCs or prevent exuberant release of histamine and other inflammatory mediators. Both histamine-2 receptor antagonists (H2RAs) and proton pump inhibitors (PPIs) are widely used in dogs with MCTs for their ability to reduce gastric acid and theoretically reduce degranulation associated adverse events (AEs). Both of these classes of acid suppressants have been found to possess a plethora of anti-inflammatory and even cytotoxic properties regarding host immunity and reduction in neoplastic cell proliferation, survival, and metastasis. Though H_2RAs are widely used in patients with MCTs to prevent tumor side effects, PPIs likely inactivate a critical proton pump, a vacuolar ATPase, that is required for MC granule structure and viability. A vacuolar ATPase inhibitor, bafilomycin A, has powerful cytotoxic effects on MCs due to granule disruption. Because PPIs also work on vacuolar ATPase pumps, we expect that routinely available PPIs, such as esomeprazole, might have similar effects. If so, use of PPIs in dogs with MCT disease might be of greater benefit than the use of H_2RAs.

In these studies, we have shown that esomeprazole, more so than famotidine, alters healthy and neoplastic *in vitro* MC structure, viability, and degranulation patterns. Treatment with esomeprazole caused a visible concentration and time-dependent increase in cytoplasmic

vacuolization via electron microscopy, as well as induced significant cytotoxicity (via both early and late apoptosis) in several species of neoplastic *in vitro* MC lines. Esomeprazole treatment, but not famotidine, also caused alterations in MC activation, as assessed by β-hexosaminidase release indicative of degranulation. An *in vitro* canine B cell lymphoma line was used as an agranulocytic control. The same treatment effects were either blunted or completely absent in the lymphoma line in comparison to all MC lines.

Although this work is *in vitro* and cannot be directly extrapolated to an *in vivo* model, we found the direct effects of esomeprazole on canine neoplastic MCs to be superior to those of famotidine, suggesting PPIs might be the acid suppressant of choice for canine MCT disease. Further studies are necessary investigating the mechanisms by which esomeprazole induces these treatment effects and if they are similar across other types of PPIs, as well as comparative, *in vivo* clinical trials investigating acid suppressant use in dogs with MCTs. Results of our work provide the framework for further investigation of efficacious use of acid suppressants in MCT disease in companion animals.

DEDICATION

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This work is solely the responsibility of the authors and does not necessarily represent any official views.

NOMENCLATURE

BafA	Bafilomycin A
BMMC	Bone marrow mast cell
BR	Canine BR mast cell
C2	Canine C2 mast cell
Caco	Cacodylate
c-Kit	Receptor tyrosine kinase
CXCL8	Chemokine receptor ligand 8
ECM	Extracellular matrix
Eso	Esomeprazole
Fam	Famotidine
FBS	Fetal bovine serum
FcƐRI	High affinity IgE receptor
GI	Gastrointestinal
Glut	Gluteraldehyde
H+-K+-ATPase	Hydrogen potassium ATPase pump
H2R	Histamine-2 receptor
H_2RA	Histamine-2 receptor antagonist
hSCF	Human stem cell factor
IL-3	Interleukin-3
IL-10	Interleukin-10
IFN-γ	Interferon gamma
LAD2	Human LAD2 mast cell
MC	Mast cell
MCT	Mast cell tumor
MOA	Mechanism of action
NGF	Nerve growth factor
PPI	Proton pump inhibitor
SCF	Stem cell factor

SubP	Substance P
TEM	Transmission electron microscopy
TIL	Tumor infiltrating lymphocyte
TNF-α	Tumor necrosis factor alpha
V-ATPase pump	Vacuolar ATP-ase pump
VEGF	Vascular endothelial growth factor

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CHAPTER I: INTRODUCTION

1.1 Acid suppressants and gastric hyperacidity

Acid suppressant medications refer to the class of drugs that inhibit the production of hydrochloric (HCl) acid from gastric parietal cells. Mechanistically, this occurs either indirectly via inhibition of acid secretagogues (e.g. molecules that stimulate acid production such as histamine, gastrin and acetylcholine [ACh]), or their receptors, or directly via blockade of the gastric parietal cell enzyme, hydrogen potassium ATPase (H+-K+-ATPase; aka proton pump). These pumps are located on the basolateral surface of the gastric parietal cell (**Figure 1.1**) and receive neurohormonal signals that stimulate increased or decreased release of hydrochloric acid (HCl) into the gastric lumen. Two well-known classes of acid suppressant drugs are the histamine-2 receptor antagonists (H_2RAs) and the proton pump inhibitors (PPIs), which made their debut onto the human medical scene in the 1970s and 1980s,[1] respectively.

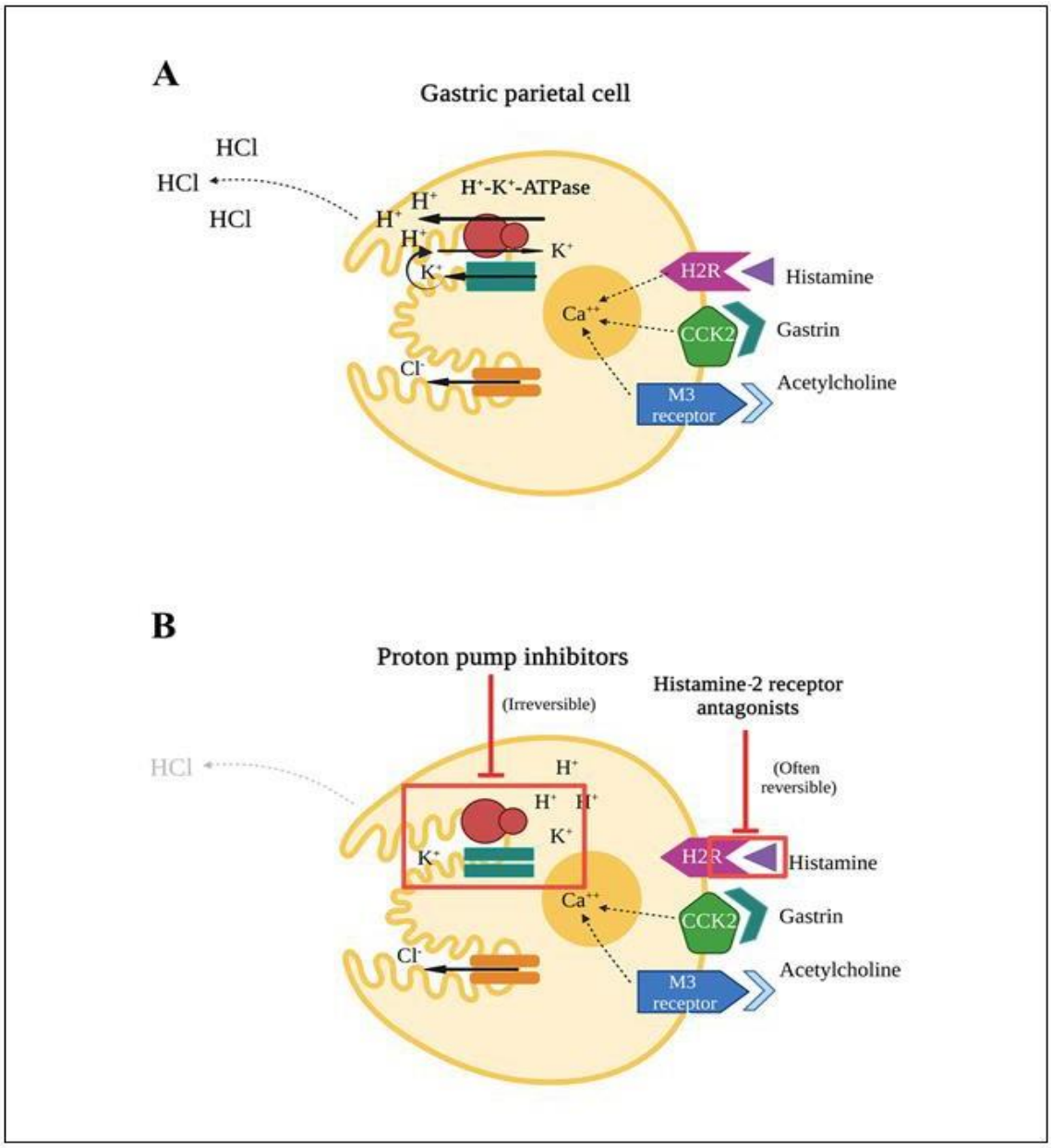

Figure 1.1 Gastric acid suppression secondary to histamine-2 receptor antagonists (H_2RAs) and proton pump inhibitors (PPIs) (created with permission via BioRender[2]). Normal HCl production from the parietal cell without an inhibitor present (A), stimulated by either histamine, gastrin, or acetylcholine. Note that the presence of either a PPI or H_2RA (B) will reduce production of HCl either via reversible blockade of the H_2R (H_2RA) or irreversible blockade of the H^+-K^+-ATPase pump (PPI).

Histamine-2 receptor antagonists (e.g. cimetidine hydrochloride, famotidine, nizatidine, ranitidine, roxatidine) competitively inhibit histamine at the histamine-2 receptor (H_2R) on the apical side of the gastric parietal cell. Famotidine and ranitidine are the most commonly used H_2RAs in both human and veterinary medicine. While histamine is the most potent acid secretagogue, it is not the only stimuli for HCl production. Gastrin and ACh are also capable of stimulating HCl production from H+-K+-ATPase pumps despite successful blockade with an H_2RA, which is one reason that H_2RAs are inferior to PPIs for reducing intragastric hyperacidity. A second reason is that H2RAs reversibly bind the H_2R and have also been shown to undergo a phenomenon called "tolerance" in humans,[3] dogs[4] and cats.[5] The amount of tolerance that develops for any given patient is governed by multiple factors, including dose, route of administration (e.g. intravenous [IV] versus oral [PO]), and frequency of administration.[6-8] Oral, but not IV, administration has been shown to induce tolerance in both cats and dogs.[4,5] Despite this, short term administration of famotidine is still superior to placebo for raising intragastric pH in both humans and companion animals,[9,10] and is still advantageous to administer to patients during the first few days of therapy if a PPI is unavailable.

Unlike H_2RAs, PPIs (e.g. esomeprazole, lansoprazole, dexlansoprazole, omeprazole, pantoprazole, rabeprazole) impart a direct, near irreversible blockade to the final step of acid production from the proton pump.[11] Thus, their mechanism of action (MOI) is independent of the type of acid secretagogue present. All PPIs contain a pyridylmethylsulphinyl benzimidazole moiety but have differing amounts of substitutions of the pyridine or benzimidazole ring structures. This results in differing potencies, and subsequent efficacies, among the types of PPIs. Following either IV, PO or subcutaneous (SC) administration, PPIs concentrate in the acidic portion of the parietal cell secretory compartment and then undergo transformation into a

cationic sulphenamide.[1] PPIs are superior to H_2RAs in their acid suppressing effects in both humans and companion animals,[9,10,12-14] and, therefore, PPIs have become standard of care for treatment of acid-related disorders (e.g. GI ulceration, bleeding, and esophagitis).

Although omeprazole (i.e. Prilosec OTC®) was the first PPI to appear on the market in the 1980s, isomers of this compound, such as esomeprazole (i.e. Nexium®) might be more efficacious for treatment of acid-related disorders. In several human studies, esomeprazole was superior in both improving clinical signs and healing of erosive esophagitis and gastroesophageal reflux disease (GERD).[15,16] While further studies are necessary in diseased companion animals, in several comparative acid suppressant studies in healthy cats[17] and dogs,[18] esomeprazole provided superior gastric suppression compared to H2RAs and other PPIs. This makes esomeprazole an attractive target for future studies investigating acid suppressant therapy for acid-related disorders in companion animals.

1.2 Anti-inflammatory and cytotoxic properties of acid suppressants

Aside from their acid suppressing effects, a plethora of human medical literature has emerged over the last 10-20 years touting the *in vitro* and *in vivo* anti-inflammatory,[19-28] cytotoxic[21,29] and even anti-neoplastic[30-35] effects of acid suppressants. Although largely derived from *in vitro* work with respect to companion animals, these findings lead us to believe that H_2RAs and PPIs confer benefits beyond their gastric acid suppressing effects to humans and animals with inflammatory or neoplastic disorders.

Treatment with specific PPIs and H_2RAs results in inhibition in the number and function of cytotoxic and tumor infiltrating lymphocytes (TILs),[36,37] neutrophils,[38,39] granulocytes,[40] and B and T cells.[41,42] The PPIs (e.g. omeprazole, lansoprazole), but not H2RAs, are able to

significantly diminish normal neutrophil migration and phagocytosis ability.[43,44] In one study, treatment with omeprazole and lansoprazole attenuated the ability of *in vitro* neutrophils to adhere to endothelial cells, thereby inhibiting a critical step in tissue inflammation.[43] Omeprazole treatment also significantly inhibited the ability of human neutrophils to phagocytize yeast compared to untreated conrols.[44] Though one of the proposed mechanisms by which PPIs might have inhibitory effects on neutrophil migration and chemotaxis is via blockade of H+-K+-ATPase or other pumps critical for cell homeostasis (e.g. vacuolar ATPase [V-ATPase] pumps; described below) on the surface of neutrophils,[39] treatment of neutrophils with a V-ATPase pump specific inhibitor failed to induce the same effects.[39] This speaks to the fact that the PPIs must alter pathways critical to leukocyte function, independent from their inhibition of the V-ATPase pump. While omeprazole has been found to restore the balance of regulatory T cells (T regs) and T helper 17 (Th17) in inflammatory tissues (i.e. children with duodenal ulcers),[26] it is unknown if this is solely from acid suppressing effects or other direct immunomodulatory actions. As with inhibition of gastric acid production, differential pH independent effects among the PPIs likely also exist. For example, in one study, esomeprazole and omeprazole had superior free radical scavenging effects in comparison to lansoprazole, pantoprazole, or rabeprazole,[45] and the differing effectiveness of PPIs seems to also hold true for anti-tumor effects.[46] While the mechanisms by which PPIs impact leukocyte function have not been elucidated, a proposed hypothesis is via their disruption of inflammatory cytokine and chemokine pathways.

Chemokine receptor ligand 8 (CXCL8), formerly known as interleukin-8 (IL-8), is a pro-inflammatory cytokine that is instrumental in creating the ideal tumor pro-inflammatory environment and serves as a stimulant for tumor proliferation, migration and metastasis.[47] This cytokine is produced by neutrophils, macrophages, monocytes, CD4 + T cells and endothelial

cells, and is a powerful chemokine for neutrophils, basophils and T cells to sites of tissue inflammation. It contributes to normal immune cell, particularly mast cell (MC), homeostasis and function.[48] Proton pump inhibitors inhibit CXCL8, in both neoplastic and non-neoplastic inflammation both *in vitro*[49] and *in vivo*.[50] For example, PPI treatment reduces CXCL8 mRNA and protein expression in the esophageal mucosa of patients with eosinophilic esophagitis,[51] as well as blocks CXCL8 production from esophageal cells that normally occurs in response to bile acid exposure.[25] Both human gastric cancer and vascular endothelial cells were found to produce significantly less CXCL8 when treated with two different PPIs compared to treatment with vehicle control.[23] PPIs also reduce or attenuate production of other important pro-inflammatory cytokines, including TNF-α and IL-1β from macrophages,[20] TNF-α in the serum and IL-6 in the tissue of colorectal cancer patients,[32] and IL-6 and TNF-α from tracheal epithelial cells.[52] It is likely that reduction in pro-inflammatory cytokines is caused by direct inhibition of proteins necessary for cytokine expression and production. For example, PPIs have been shown to directly inhibit the function of the transcription factor, nuclear factor kappa beta (NF-κβ),[25] which is essential to production of CXCL8.

As previously mentioned, one of the theories behind how PPIs might be inducing these cytotoxic and anti-inflammatory effects is via inhibition of the vacuolar ATPase (V-ATPase).[53] These V-ATPase pumps are found in both healthy and neoplastic cells, and are responsible for proton transport and the maintenance of ideal pH gradients between intra and extracellular compartments.[54,55] These pumps contain both peripheral (V_1) and integral (V_0) domains, which are responsible for ATP hydrolysis and proton exchange, respectively. **Figure 1.2** depicts the pH gradient established by a V-ATPase pump. Although healthy cells use V-ATPases to carry out normal functions, certain tumors also use these pumps to establish an acidic tumor

microenvironment, increase angiogenesis and metastasis, and even acquire chemotherapy resistance.[55,56] It is thought that more aggressive, neoplastic phenotypes upregulate these pumps in order to establish a neutral intracellular, but overly acidic luminal and extracellular compartments. The acidic tumor microenvironment recruits pro-inflammatory mediators, proteases responsible for tissue matrix degradation and also upregulates pro-angiogenic factors (e.g. VEG-F and CXCL8) critical for tumor metastasis.[57-59] This pH gradient also allows for the trapping of certain chemotherapeutic agents in spaces where they cannot penetrate, and effectively kill, the neoplastic cell. Disruption or blockade of these pumps by selective V-ATPase pump inhibitors, such as bafilomycin A (BafA), has been shown to both directly induce tumor cell apoptosis and reduce the metastatic potential of certain tumors.[60] One proposed mechanism of apoptosis induction is via promotion of intracellular acidity and reduction in pH buffering capacities.[61] It is unknown, but likely, that PPIs induce tumor cell death via inhibition of V-ATPases. Indeed, omeprazole, albeit to a lesser extent than bafilomycin A under physiological conditions, binds to renal and bone V-ATPases[62] in healthy cells. While the MOA by which PPIs induce their anti-neoplastic effects is likely multi-factorial, it is unclear how much and in which tumor types the inhibition of V-ATPases plays a pivotal role. Multiple *in vitro* studies have demonstrated that PPIs have the ability to cause apoptosis and inhibit proliferation of selective neoplastic human cell lines.[34,63] Comparative, retrospective studies investigating the use of acid suppressants in conjunction with chemotherapeutics show that human patients receiving famotidine and lansoprazole as part of their treatment regime exhibited reductions in pro-inflammatory cytokines, with an increase in tumor cell apoptosis and clinical response seen in the PPI treated groups.[63] Proton pump inhibitors, specifically, have also been linked with increased response rates in human cancer patients.[64]

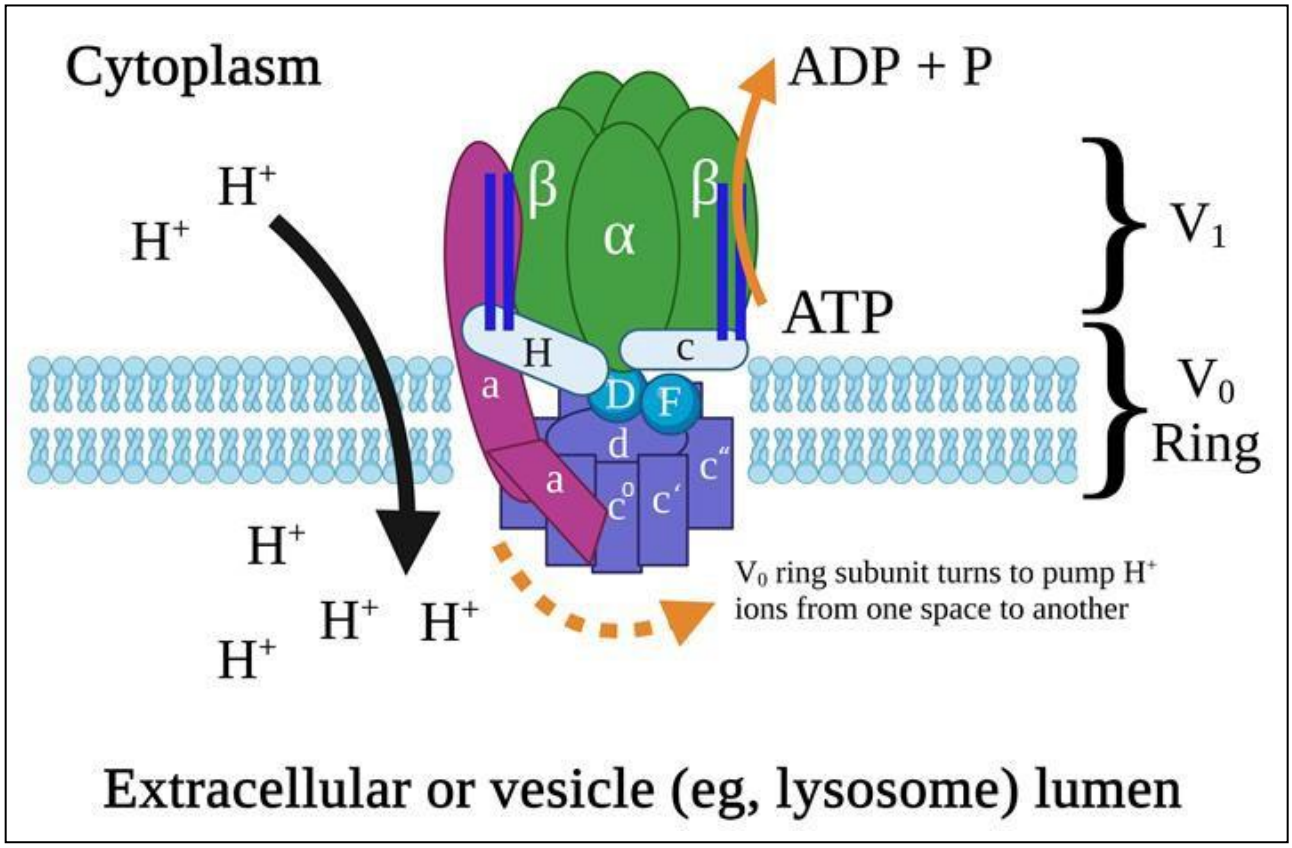

Figure 1.2 Vacuolar-ATPase (V-ATPase) pump including the V_1 and V_0 ring subunits (created with permission via BioRender[2]). The V_1 subunit is composed of the alpha (α), beta (β), D, F, H, and c units, as well as the units responsible for hydrolysis of ATP into ADP and phosphate (blue units connected to H and c). The V_1 and V_0 subunits are connected by unit a, which allows for rotation of the V_0 ring in order to transport hydrogen ions (H^+) from the cytoplasm into either a vesicle or the extracellular space. The V_0 subunit is composed of units d, c^0, c', and c", and connected to V_1 via the a unit.

Selective H_2RAs also seem to possess some anti-inflammatory properties, specifically in decreasing pro-inflammatory cytokine release from various peripheral blood mononuclear cells (PBMCs),[41] murine T cells and bone marrow derived dendritic cells,[40] human leukemic MCs,[65] and basophils.[65] Unlike PPIs, there is no literature to support that H_2RAs directly kill neoplastic cells or alter the tumor microenvironment by modulating extracellular pH. Some compelling

studies have shown that H_2RAs improve the anti-neoplastic action of peripheral blood mononuclear cells (PBMCs) and tumor infiltrating lymphocytes (TILs).[37] Tumor infiltrating lymphocytes excel at specifically killing tumor cells. One study documented that the addition of famotidine, at concentrations meant to mimic the plasma concentration of the drug in blood, both enhanced the cytotoxic activity of PBMCs and TILs.[37] The MOA by which famotidine was able to exert these effects this was not determined from this study. H_2RAs likely have a bigger role in modulating cytokine profiles in the gut, via action on H_2 receptors found throughout the small intestine. One example would be the effect H_2RAs have in antagonizing the immunomodulatory effects of histamine on intraepithelial lymphocytes (IELs). Histamine helps to regulate cytokine profiles in the gut with increased histamine concentrations leading to reductions in TH1 cytokine (i.e. IFN-γ, TNF-α and IL-2) production.[66] H_2RAs, but not H_1R or H_3/H_4R antagonists, were found to reverse the effects of histamine on IELs. H_2RAs also modulate immune responses and cytokine profiles in response to bacteria in the gut of human patients with IBD.[67] While the MOA by which H_2RAs exert these effects is likely different from those of the PPIs, further investigation of the use of both classes of acid suppressants in inflammatory and certain neoplastic disease states is warranted.

In companion animals, the most common uses of acid suppressants are for disorders related to GI hyperacidity (e.g. GI ulceration and bleeding, esophagitis, GERD) and mast cell tumor (MCT) disease. Mast cell tumors are the most common skin tumor of dogs worldwide, and these tumor cells contain large amounts of pro-inflammatory cytokines and vasoactive amines (e.g. histamine, heparin) within intracellular granules. Because MCs have a critical role in antimicrobial, antiparasitic and allergic responses, they are able to release these intragranular compounds during a process called degranulation. Though there is certainly a beneficial purpose

to the host following degranulation, there are also many adverse effects (AEs) that might ensue, especially with neoplastic MCs. Examples of these AEs include anaphylaxis with vasodilatory shock, urticaria, hives, and GI ulceration secondary to gastric hyperacidity from excess circulating histamine. The rationale for the use of acid suppressants in MCT disease is to combat the sequelae of GI ulceration. Acid suppressants are often used in conjunction with histamine-1 receptor antagonists (H_1RAs) in the treatment of pets with MCT disease. Despite this, there is limited to no evidence in the veterinary literature that acid suppressants actually have beneficial effects in companion animals with MCT disease. Investigation of the effects of acid suppressants on *in vitro* or *in vivo* MCs, healthy or neoplastic, has not yet been performed. If acid suppressants are found to have similar effects on canine MC function and viability as described above for other immune cells, especially if one drug is superior to another, this provides rationale for investigation of specific mechanisms of the effects, as well as *in vivo* study of these drugs in companion animals.

1.3 Properties of healthy and neoplastic mast cells across multiple species

Mast cells (MCs) are $CD117^+$/Fc epsilon (FcƐ) $receptor^+$ eukaryotic, granulocytic cells whose progenitors originate in the bone marrow (BM).[68] Following release from the BM, MCs migrate to a target organ system, usually mucosal in nature (e.g. gastrointestinal [GI], respiratory, ocular), where they then undergo maturation and differentiation governed by tissue-specific, local growth factors.[69] These cells are best known as first line effector cells in Type I allergic and hypersensitivity reactions (e.g. asthma, rhinitis, urticaria, anaphylaxis). The presence or absence of stem cell factor (SCF) secreted by endothelial cells and fibroblasts in the tissue microenvironment dictates MC maturation. Binding of SCF to the SCF-specific surface receptor, KIT (e.g. CD117), initiates MC maturation and survival.[70] Mature MCs are often structurally

recognized for their large, intracellular granules, which contain an array of biogenic amines (e.g. histamine, serotonin), pro-inflammatory cytokines (e.g. tumor necrosis factor α [TNFα],[71] interleukins [ILs]),[48] lysosomal proteins (e.g. acid hydrolases [β-hexosaminidase]), critical immune regulatory molecules (i.e. major histocompatibility complex class II [MHC class II])[72] and other compounds such as heparin. Many of these molecules are synthesized and released *de novo* as well as via the act of degranulation.[73] Another important role of SCF is enhancement of MC pre-formed and *de novo* synthesized mediator release.[70] In the presence of bound SCF, the KIT/CD117 receptor is dimerized, which then initiates a cascade of protein phosphorylation that in the presence of intracellular calcium (Ca^{2++}), leads to transcription of molecules important for biological MC effector functions.[68]

Mast cells can be classified into subtypes according to their tissue phenotype (e.g. mucosal or connective tissue), the presence or absence of intracellular serine proteases (e.g. tryptase, chymase), and their response to different types of degranulation stimuli. Classification schemes vary depending on the species in question, but characterization according to protease content is usually as follows: tryptase$^+$/chymase$^+$ (MC_{TC}), tryptase$^+$/chymase$^-$ (MC_T), and tryptase$^-$/chymase$^+$ (MCc). Canine mast cell tumors (MCTs) can be characterized based on this protease scheme.[74] Protease content and tissue phenotype varies depending on the species of interest, meaning that tryptase and chymase are present in differing amounts in human versus rodent mucosal and connective tissue MCs. A variety of MCs have been isolated from rodents (e.g. mouse bone marrow mast cells [BMMCs], rat intestinal mucosal [IMMCs],[75] peritoneal mast cells [PMCs], rat basophilic leukemic cells [RBL-2H3]), humans (e.g. LAD1 and 2,[76] HMC-1,[77] LUVA[78]), and dogs (e.g. BR,[79] bone marrow mast cells [BMCMC],[80] C1,[81] C2, MPT-1, canine G cell), with varying degrees of differentiation. A summary of the characteristics

specific to these MC lines can be found in **Table 1.1**. Some *in vitro* MCs can be manipulated to grow as either a mucosal or connective tissue phenotype depending on which cytokines and growth factors are present in co-culture. An example of this would be the BMMC line, which takes on a mucosal phenotype in the presence of IL-3. This can be useful for comparative studies looking at different MC phenotypes from the same species.

Species	Cell line	Characteristics	Advantages	Limitations
Human	HMC-1. Developed from peripheral blood of human patient with MC leukemia.	Non-adherent; require serum free media; have different types of c-kit activating mutations depending on if looking at HMC-1.1 or 1.2, which results in SCF-independent phosphorylation of CD117 (which might contribute to proliferation of these cells)	Fast growing cell line with short doubling time	Major disadvantages are HMC-1 have decreased granularity (phenotypically do not resemble MCs) and they lack high affinity IgE (FcER1) receptor. So not good model for IgE mediated degranulation.
	LAD2 cells. Originated from human bone marrow cells (patient with mastocytosis).	Non-adherent; media must be "hemidepleted" when changed; require serum free media; lack c-kit mutation; express FcER1, CDs 4, 9, 13, 22, 45, 64, 71, 103, 117, 132, 184 and 195; histamine content per cell is ~ 3.1 pg/cell	Resemble CD34+ derived human MCs, have functional FCER1 and FcγR1 receptors; contain tryptase, chymase and histamine; IgE mediated β-hexosaminidase release of ~ 40%; thought to resemble a more mature phenotype based on slower doubling times	Does not have mutation in c-kit, so must be supplemented with hSCF; slow doubling time (~ 2-3 weeks to double); some papers cite they are not good model for study of MC cytokine release
	LUVA cells. Derived from CD34+ enriched mononuclear cells from human donor.	Display high amounts of c-kit and FcER1; no mutations in Kit; highly granulated; tryptase (+)	Can be maintained without the addition of SCF to culture media; morphologically resemble mature MCs; degranulate in response to IgE/can be used as model for "natural" degranulation	Major disadvantage is that they can lose their FcER1 over time when maintained in culture for long periods of time
Rat and mouse	Rat basophilic leukemic cells (RBL-2H3). Formed by injecting rats with chemical carcinogen B-chlorethylamine	Adherent growth; high histamine content in cells; have SNARE proteins for exocytosis; have c-kit receptor (with mutation in c-kit) and high affinity IgE (FcER1) receptor; serotonin is critical mediator	Fast doubling time; very well established model for IgE mediated degranulation; high histamine content in cells; very well accepted model for studies of exocytosis; Do have c-kit mutation, so similar to MCs in this way	Have many characteristics that also resemble basophils rather than MCs; visually, they grow more like fibroblasts than MCs; have TLR4 but NOT activated by LPS
	Murine bone marrow derived mast cells (BMMCs). Taken from progenitor cells in mouse bone marrow.	Non-adherent; have functional high affinity IgE (FcER1) receptors; no mutations or genetic modifictions	High yield of cells from mice femurs; significantly cheaper than human MCs; can be model for either mucosal or serosal type MCs depending on which cytokines or growth factors are supplemented (IL-3 promotes mucosal phenotype, whereas SCF promotes serosal type); one big application is study of critical signaling events in receptor mediated MC activation	Slow growing at first, takes about 4 weeks to start generating functional BMMCs
	Rodent peritoneal mast cells (PMCs). Obtained from collection of MCs via peritoneal lavage of mice.	Highly granular; express high affinity IgE receptors/FcER1	Morphologically resemble mature mast cells as they have already matured in the periphery (ie, not coming from marrow); highly granular; good model for connective tissue phenotype; can be used very quickly (within 4 days of obtaining)	Can be hard to obtain high numbers of mast cells (not going to get as high of yield as from marrow)
Canine	Neoplastic canine C1 line. Isolated from skin of dog with mastocytoma early in the course of disease (single excisional biopsy).	Non-adherent; possess more histamine than canine C2 cells (~ 0.46 pg/cell); contain functional high affinity IgE FcER1 receptor; tryptase (+)	Good model for study of "natural" IgE mediated degranulation; granulated; morphologically resembles mature MCs; Fast doubling time, can generate a large amount of cells in short time period; stable in regards to growth patterns, histamine content after 55 passages.	Relatively low histamine content compared to some other in vitro MC lines
	Neoplastic canine C2 line. Developed from cutaneous dog mastocytoma. This is same dog that C1 was derived from, but was isolated at end stage/disseminated disease when dog considered to have mast cell leukemia.	Adherent @ low conc, non-adherent @ higher conc; have internal tandem duplication in JMD mutation in Kit; have FcER1 receptors, respond to IgE; both chymase and tryptase (+), which differs slightly from C1 line ; low histamine content compared to other cell lines (~0.07 pg/cell)	Can be used as model for IgE mediated/"natural" degranulation; fast doubling time; do not require cytokines or SCF for growth; morphologically resembles mature MC; considered a well differentiated, mature canine MC line; Fast doubling time, can generate a large amount of cells in short time period; stable in regards to growth patterns, histamine content after 55 passages	Not as well granulated as some other in vitro MC lines (less granulated than C1); less histamine content than some other in vitro MC lines
	Canine MPT-1 line. Isolated from connective tissue MCT in a dog.	Non-adherent; found to LACK mutations in c-kit; SHORT doubling time (~40 hr); high amount histamine in cytoplasm (> HMC, BR); both IgE FcER1 and wild type c-kit receptors; chymase (+); contain histamine (~ 0.82 pg/cell)	One of few lines to contain wild type c-kit receptors; one of best canine connective tissue MCT models; do not need growth factors in media; can be used as model for IgE mediated/"natural" degranulation and also degranulate in response to A23187	Weaker degranulation with cmpd 48/80 and Substance P, so need to be aware of this
	Neoplastic BR canine line	Non-adherent; has activating point mutation in JMD of Kit; low histamine content compared to other MCs (~ 0.04 pg/cell)	Fast doubling time; good model for mediators released by non-immunological activation; no need for cytokines, addition of growth factors to media	Lack FcER1, so cannot b used as model for IgE mediated degranulation; considered poorly differentiated in vitro canine MC line
	Canine bone marrow derived cultured mast cells (BMCMC); taken from dog bone marrow CD34+ cells	Non-adherent; contain chymase, tryptase; express c-kit, FcER1; also produce IL-8, MCP-1; need serum free media with SCF supplementation; no need for IL-3 for differentiation; contain histamine (~ 1 pg/cell)	Appropriate model for study of MCs from canine skin (as both tryptase, chymase +); phenotypically resemble MCs with round shape; good model for study of normal/non neoplastic MCs; might resemble human more than murine MCs given no need for IL-3	Have to be cultured with SCF; not good for study of neoplastic mast cell behavior
	Canine neoplastic G line. Isolated from dog skin mastocytoma.	Non adherent; heavily granulated with more electron dense granules than C1, C2 lines; larger in sizes than C1 and C2 lines; contain histamine (~1.29 pg/cell)	Contain large amounts of histamine, so might be good model for looking at histamine release following activation; morphologically mature MC	Might not be best model for IgE mediated degranulation as response to IgE stimulation did not consistently produce activation like C1, C2 lines

Table 1.1 Characteristics of relevant rodent, human and canine *in vitro* mast cell lines.

The composition of MC granules, like many other factors, varies on both an intra-individual and interspecies level. For example, three specific types of granules have been identified in murine BMMCs[72]: type I granules (i.e. MHC II, β-hexosaminidase, lysosomal associated membrane protein [LAMP]-1 and 2), type II granules (i.e. same as type I but also including serotonin), and type III granules (i.e. β-hexosaminidase and serotonin only). Within canine MC lines, the G cell line has at least two different granule subtypes characterized by differing amounts of proteases, whereas granules in the BR line lack heterogeneity.[82] Not all MCs have been as well characterized as the BMMCs and LAD2 cell lines, so it is possible that a variety of other granule types and compositions have yet to be discovered. Importantly, the composition of an individual MC's granules matters as these molecules are released during degranulation and then have subsequent effects on the host, which is a crucial part of the MC's immune and allergic response. The specific type of mediator(s) released from the MC is especially important to take into consideration when thinking about how to assess MC function based on *in vitro* models and which *in vivo* disease states those models best recapitulate.

1.4 Stimulators of mast cell degranulation, and degranulation sequelae

A variety of different secretagogues and pathways are responsible for MC degranulation, but activation is typically divided into two broad categories: immunoglobulin E (IgE) versus non-IgE mediated.[83] Mast cells possess both high and low affinity IgE receptors, FcƐRI and FcƐRII, respectively.[84] The amount of FcƐRI present on the MC surface is a dynamic process, and is dependent on how much circulating IgE is present (e.g. more local IgE results in a higher amount of surface FcƐRI).[70] Mechanistically, IgE-mediated degranulation occurs when there is cross-linking of the FcƐRI receptor with IgE bound to antigen/allergen (**Figure 1.3**). This receptor cross-linking in turn sets off an intracellular signaling cascade of protein

phosphorylation and, ultimately, Ca^{2++} efflux from the endoplasmic reticulum that has an end result of increasing the free intracellular Ca^{2++}. This increase in Ca^{2++} eventually facilitates granule-to-granule or granule-to-plasma membrane fusion and the eventual act of anaphylactic degranulation.[68,73] Another type of mediator release is "piecemeal" degranulation, which is the more gradual release of mediators without granule fusion.[73] The type of degranulation is largely regulated by the strength and duration of the stimulant,[68] but is more commonly observed with toll like receptor (TLR) stimulated pathways.[73] Both types of degranulation have been reported to occur *in vitro*, *in vivo* and *ex vivo* in human, mouse and rat models.[73,85,86] Specific differences in degranulation pathways have yet to be investigated in in vitro or in vivo models of canine MCs.

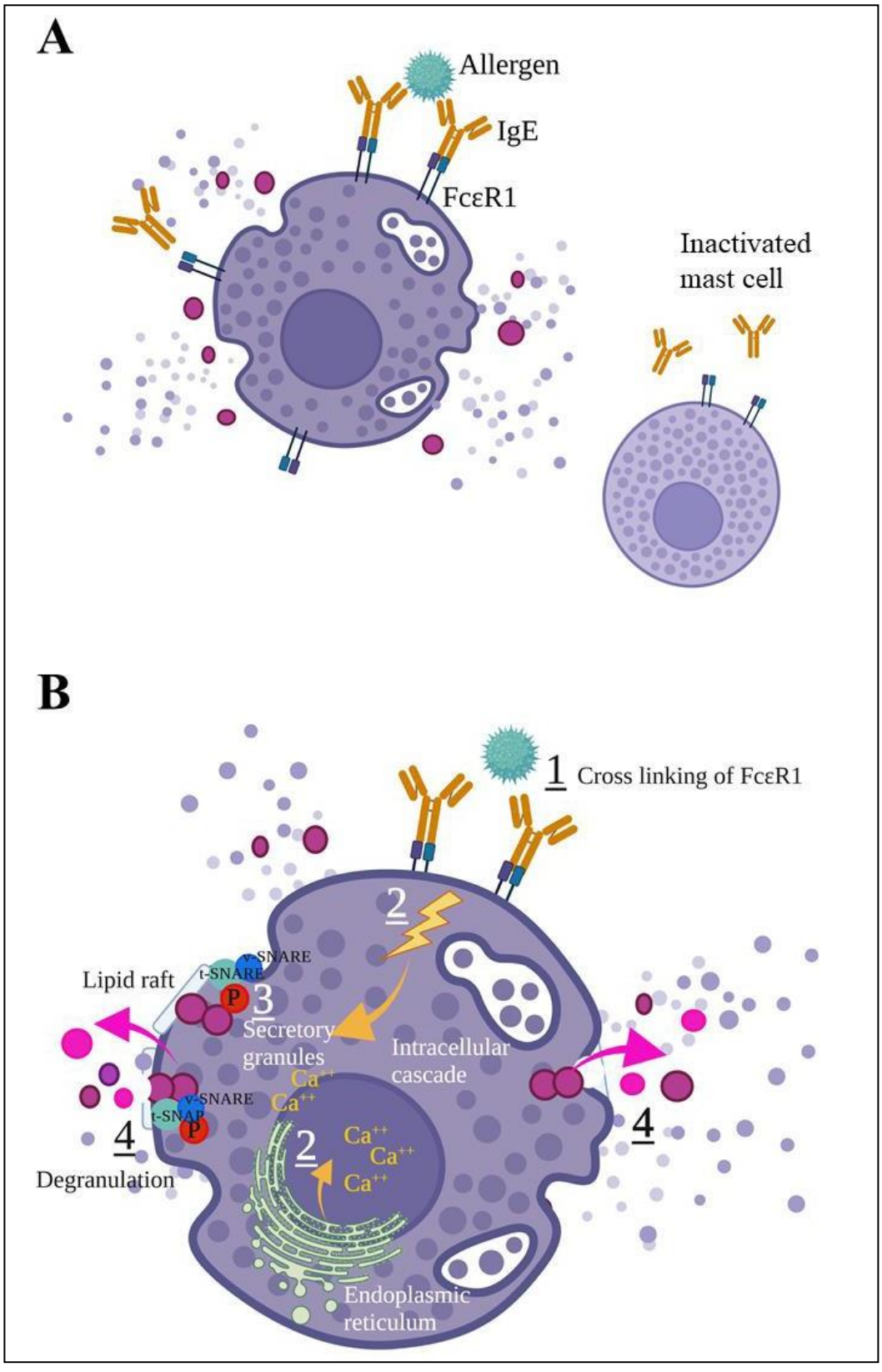
A
Allergen
IgE
FcεR1
Inactivated
mast cell
B
1 Cross linking of FcεR1
2
v-SNARE
t-SNARE
Lipid raft
P
3
Secretory
granules
Intracellular
cascade
Ca++
Ca++
v-SNARE
t-SNAP
P
4
Degranulation
2
Ca++
Ca++
Ca++
4
Endoplasmic
reticulum

Figure 1.3. Immunoglobulin-E (IgE) mediated mast cell activation and degranulation (created with permission via BioRender[2]). (A) Inactivated (lower right) mast cell, as well as mast cell with bound IgE in its high affinity receptor, FcƐR1 (upper left). Note the allergen (i.e. green pollen) cross linking the bound IgE. (B) Intracellular cascade stimulated by cross linked IgE receptors, culminating in MC degranulation. Following cross linking of the IgE receptors (1), an intracellular signaling cascade begins that includes release of intracellular calcium via the endoplasmic reticulum (2). The release of intracellular calcium triggers secretion through conserved cellular mechanisms (3) involving the calcium--sensing protein synaptotagmin, and t-SNAREs (syntaxin and SNAP23) located on the plasma membrane, together with v-SNAREs (synaptobrevin) located on the granule membrane to facilitate MC degranulation (4).

Main stimulants of non-IgE mediated degranulation include neuropeptides (e.g. corticotropin-releasing hormone [CRH], substance P [SubP]),[83,87] nerve growth factor (NGF),[87] vasoactive intestinal peptide (VIP),[73,87] immunoglobulin G (IgG),[83] antimicrobial peptides, and complement mediated activation.[83] Many of these stimulants act via TLR-mediated pathways or via specific surface receptors on the MC. One of the main known differences between IgE and non-IgE mediated degranulation events is that IgE-mediated events nearly always result in a visible ultrastructure change in granules (e.g. fusion with other granules or plasma membrane),[86] whereas as other types of degranulation often do not. *In vitro* degranulation has been successfully induced in human, rodent and canine MC lines.[76,79,88-90] For *in vitro* models, both physiologic (e.g. IgE dinitrophenyl [DNP], SubP, concavalin A)[76,79] and non-physiologic (e.g. calcium ionophore A23187[91] and compound 48/80[91,92]) MC stimulants are available, but only physiologic stimulants mimicking *in vivo* mediator release. The use of A23187 and compound 48/80 are often performed as positive controls that reliably induce degranulation. The development of these models has made *in vitro* study of compounds that affect MC function (e.g. mediator formation and release) possible.

An important sequelae of MC degranulation is the release of both pro and anti-inflammatory cytokines and chemokines, along with molecules capable of extracellular matrix

(ECM) breakdown. Pro-inflammatory molecules identified as synthesized and released by MCs[93,94] of multiple species include tumor necrosis factor-alpha (TNF-α), ILs-1β, 6, 17, leukotrienes (LTs) B_4, C_4, D_4, macrophage inflammatory protein-1 (MIP-1),[94] prostaglandin D_2 (PGD_2), chemokine ligands (CCLs)-2-5, 13, 20, and chemokine receptor ligand-8 (CXCL-8). Anti-inflammatory cytokines and chemokines secreted by MCs include interferon-γ (IFN-γ), IL-4, 13 and 10.[93] These molecules have significant downstream effects on leukocyte (e.g. basophil, eosinophil, lymphocyte, monocyte, neutrophil) migration and tissue trafficking, which contributes to the severity of the inflammatory response. While many of these mediators are released during both anaphylactic and piecemeal degranulation, some are released independent of MC activation.[95] Examples of this include CXCL-8[95] and MIP-1,[95] suggesting that the mere presence of dormant MCs can influence the local tissue inflammatory environment. This might make the organ systems in which MCs reside (e.g. mucosal tissue-environment interfaces) more prone to inflammatory, allergic, or anaphylactic responses. Because an effective inflammatory response relies not only on immune cell recruitment, but entry into the target tissue of interest, MCs also secrete a variety of enzymes capable of destroying ECM.[96] Tryptase, chymase, capsethin G, and MMP-9 are secreted by activated MCs, which allow for tissue remodeling and the entry of inflammatory cells into a given site.[96-98]

Ultimately, the synthesis, storage, and release of the MC inflammatory mediators depend on local, environmental factors and the presence of MC stimulators. Similarly, the presence or absence of and severity of MC degranulation depends on the amount and type of stimulant. This is important to take into consideration when evaluating any *in vitro* or *in vivo* model for evaluating MC function.

In addition to inflammatory mediators, MCs also store and release many cytokines, chemokines, proangiogenic molecules, and growth factors capable of nourishing tumor microenvironments. Therefore, depending on the type of neoplasm, inhibition of MC function might improve morbidity and mortality in select patient populations. Specific cytokines identified as instrumental in creating a favorable tumor microenvironment are TNF-α, IL-6, and matrix metalloproteinase-9 (MMP-9).[99] Following release of IL-6, MCs are able to shift the host T regulatory phenotype into a pro-inflammatory cell type which then produces IL-17. Some of the growth factors most advantageous for neoplastic cell survival include vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), SCF and nerve growth factor (NGF).[100] These factors enhance neoplastic cell survival, proliferation, and metastasis. Additionally, dysfunction of host T cells and natural killer (NK) cells can occur both from the acidic tumor microenvironment as well as from factors directly released from the MC. Examples of this include the ability of histamine to stimulate recruitment of monocytes as well as promote both T helper cell type 1 and 2 responses, perpetuating inflammation.[68] Mast cell-mediated tryptase release can also stimulate cyclooxygenase (COX) production from neoplastic cells.[101] Certain neoplasms seem to display a symbiotic relationship with MCs by their ability to secrete SCF and cyclooxygenase (COX), which in turn enhances MC growth and proliferation in the tumor microenvironment.[101] Secretion of COX via tumor cells in turn stimulates production of VEGF from MCs, stimulating angiogenesis.[101]

1.5 Neoplastic mast cell disease in humans and dogs

Neoplastic MC disease differs in terms of pathogenesis, clinical presentation, and prognosis between species. In humans, MC disease is divided into either the cutaneous (CM) or systemic (SM) manifestations,[102] and then further categorized by the World Health Organization

(WHO) into 7 sub-categories.[103] Dogs also develop cutaneous or systemic manifestations of MC neoplasia, and similar to humans, most arise from mutations in the KIT gene. There is no age or sex predisposition in dogs, but certain breeds (e.g. boxers, Boston terriers, Chinese shar-pei) are overrepresented for MCT disease, with boxers and Boston terriers comprising up to 50% of affected dogs in some studies.[104] The CM in humans tends to be more benign, and phenotypically differs from mast cell tumors (MCTs) in dogs. The most common manifestation of the CM in humans is urticaria pigmentosa,[102] whereas dogs usually develop well demarcated cutaneous or subcutaneous (SC) tumors. In dogs, MCTs are the most common skin neoplasm, accounting for 7-21% of skin tumors worldwide.[105] Additionally, dogs develop metastatic MCT disease, but metastasis is much less common than in humans. Aggressive, systemic mastocytosis is seen in both children and adults, although children are more often affected by the less aggressive CM.[102] e.g.

Due to the array of pro-inflammatory, vasodilatory, and allergenic molecules contained in MCs, life threatening adverse effects (AEs) secondary to MCT degranulation include anaphylaxis, vasodilatory shock and gastrointestinal (GI) ulceration. There are very few studies in which the incidence or prevalence of any of these AEs in companion animals have been reported. One of the only studies to investigate for GI ulceration looked at both naturally occurring mastocytosis in client-owned dogs, along with artificially induced MCT disease in Beagle dogs.[106] Twenty-four dogs with cutaneous or SC MCTs had necropsies performed and significant findings included gastric or small intestinal erosions (n=20/24 dogs), with most dogs having gastric lesions and none having only one solitary lesion. In order to determine if healthy dogs were susceptible to ulcerogenic effects of excess histamine, Beagle dogs were given SC injections of either histamine (n=5) or combination histamine + heparin (n=4) for anywhere from

1-4 months, then humanely euthanized and necropsied. Pathology of the GI tract showed that all dogs receiving histamine only had gastric, but not duodenal, lesions. Dogs receiving combination therapy had no lesions. All dogs with naturally occurring MCTs had more severe lesion scores than the experimentally-induced group. Weaknesses of this study included a lack of investigation for co-morbidities contributing to GI lesions (e.g. primary GI disease, hepatic or renal disease), omission of an age-matched control group, and quantification of systemic or local (e.g. GI tissue) histamine. While further studies with age-matched controls are necessary to investigate for the presence of AEs in metastatic and non-metastatic canine MCT disease, in this study, dogs with even locally invasive MCTs were found to have GI lesions[106] This finding suggests that treatment with antihistamines and potentially acid suppressants is in fact warranted regardless of the presence or absence of metastasis.

Following surgical resection of the tumor, options for incompletely excised or metastatic MCT disease in dogs includes single or multi-agent therapy with chemotherapy (e.g. vinca-alkaloids, tyrosine kinase [TK] inhibitors, alkylating agents) and/or prednisone. Combination vinblastine and prednisone therapy have resulted in the most favorable median survival times (MSTs) according to the veterinary literature (CITE), although more and more research evaluating the efficacy of TK inhibitors (e.g. Palladia) is underway. Steroids are thought to exert anti-tumor effects via glucocorticoid (GC) receptors found in the cytosol of canine MCTs. In general, prednisone exerts most of its anti-tumor effects through inhibition of tumor cell growth and reproduction. At least one study has demonstrated *in vitro* and *in vivo* inhibition of cutaneous canine MCT proliferation and induction of MCT apoptosis.[107] Additionally, steroids help decrease the inflammatory tumor microenvironment by reducing peritumoral edema and the production of systemic pro-inflammatory cytokines. Specifically, steroids can decrease

circulating levels of T lymphocytes, inhibit neutrophil, macrophage and monocyte migration and reduce production of interferon (IFN). Therefore, it makes sense that prednisone has been a mainstay of therapy for canine MCTs. Importantly, a recent paper found that chronic steroid treatment of *in vitro* canine neoplastic MCs resulted in steroid-resistant populations.[108] It is unknown if this same phenomenon occurs *in vivo*, but if it does, investigation of novel therapeutic strategies, such as acid suppressant drugs, to either reduce tumor associated inflammation or directly kill neoplastic MCs would be beneficial.

1.6 *In vitro* models for the study of neoplastic mast cells in dogs

Creation of *in vitro* models for canine MCT disease has been challenging due to the difficulty in isolating primary neoplastic MCs from dogs; however, a handful of well and poorly differentiated and characterized MC lines isolated from other species exist. Probably the best characterized lines include the LAD2 line, isolated from a human with MC leukemia,[76] the BMMC line, taken from the bone marrow of healthy mice,[109] and the RBL-2H3 line, derived from a rat with basophilic leukemia. While the RBL-2H3 line is technically not a true MC line, it is one of the most well-established *in vitro* models for the study of MC degranulation, given that basophils degranulate in response to similar stimuli and contain high amounts of histamine and easily quantifiable β-hexosaminidase.[110] Characteristics of importance to consider when evaluating selection of the correct cell line for study of MC function would be the types of surface receptors present (e.g. FcƐR1), the protease content of granules, what quantifiable intracellular molecules exist (e.g. histamine, β-hexosaminidase), and if cell growth is dependent on cytokines and/or growth factors. Cells with the high affinity FcƐR1 are more likely to phenotypically represent *in vivo* MCs capable of undergoing IgE mediated degranulation. Both

the LAD2 and murine BMMC lines lack KIT mutations and therefore require the addition of SCF as well as IL-3 (BMMC only) for normal growth.

Two neoplastic canine cell lines, C2[111] and BR,[112] have been characterized and used for the *in vitro* study of canine neoplastic MC behavior and the effects of potential novel therapeutics. Both lines were isolated from different dogs with cutaneous MCTs (mastocytomas), and initially propagated in nude mice prior to successful maintenance and passage *in vitro*.[111,112] The C2 line is considered well-differentiated and contains both tryptase and chymase. These cells have been determined to have surface IgE receptors, although the functionality of these receptors is questionable given inconsistent degranulation in response to IgE.[90,111] The BR cell line is considered to be less differentiated and an altogether "immature" neoplastic cell in comparison to the C2s, which is evident in the number and appearance of their granules.[79] Both lines contain varying amounts of histamine, and have been shown to degranulate in response to calcium ionophore A23187 (both),[111] SubP,[79] compound 48/80 (BR only)[79,111] and IgE (C2 only).[112] The BR cell line is thought to lack functional FcƐR1 receptors, and unfortunately are also not good models for IgE-mediated degranulation. Both C2 and BR have also been used for investigation of the effects of certain drugs on MC ultrastructure (e.g. light microscopy, transmission electron microscopy [TEM]).[79,112] Importantly, both of these cell lines proliferate without the addition of SCF or cytokines due to KIT mutations,[113-115] mimicking *in vivo* canine MCT disease.

1.7 Rationale for investigation of acid suppressants in canine neoplastic mast cells

Although studies investigating the anti-inflammatory and cytotoxic effects of acid suppressants on healthy or neoplastic MCs are limited, a few studies have shown the effects of both V-ATPase specific inhibitors (e.g. BafA) and PPIs (i.e. omeprazole) on *in vitro* murine

MCs. One of the first studies investigating treatment of murine bone marrow mast cells (BMMCs) with BafA showed that inhibition of this pump resulted in altered cell morphology (**Figure 1.4**), reduced tryptase enzyme activity and altered intracellular histamine content.[116] This study was also the first to prove that an acidic pH was important to MC granule homeostasis, as intracellular pH was altered along with MC function following BafA treatment. The results of this study lead investigators to believe that alteration of MCT intracellular pH is a very attractive therapeutic target. A follow up study looked at omeprazole treatment of the same *in vitro* BMMCs, along with cord derived human MCs.[117] Results of this study mirrored the Pejler et al study, and demonstrated that omeprazole treatment altered IgE-mediated degranulation and reduced both cytokine and histamine release in response to allergen stimulation.[117] While the exact MOA by which omeprazole induces these effects remains unknown, one hypothesis is via inhibition of V-ATPase pumps. It has not yet been investigated if similar effects occur when *in vitro* canine MCs are treated with H2RAs or other types of PPIs.

Because published veterinary literature justifying the use of acid suppressants in MCT disease does not exist, a standardized protocol for the use of acid suppressants in canine MCT disease is also lacking. Many veterinarians choose to use both PPIs and H2RAs together, especially in dogs with metastatic disease with hopes that they might have a synergistic effect. Although both drugs likely provide benefits, PPIs and H2RAs should not be used concurrently when reduction of gastric acid secretion is also desired, as in dogs with MCT.[10] PPIs accumulate and are activated in the acidic environment of the parietal cell. Concurrent use of H_2RAs decreases the gastric acid suppressing effect of PPIs by decreasing parietal cell acidity and thus parietal cell accumulation of PPIs; thus, clinicians must choose the more desirable acid suppressant. Although PPIs are superior to H_2RAs for increasing gastric pH in dogs, H_2RAs are

considered to be standard of care for dogs with gross MCT disease with the rationale that they will better mitigate the more systemic effects of histamine release. In addition to some of the anti-neoplastic benefits already mentioned, there is also *in vitro* support that PPIs might directly impact MC viability and function. Although this has been investigated with omeprazole, treatment effects of potentially more efficacious PPIs such as esomeprazole has yet to be performed. As does the investigation of these effects on canine neoplastic MCs *in vitro*. Therefore, investigation of the differential anti-inflammatory and cytotoxic effects of acid suppressants on canine MCT disease is warranted.

1.8 Hypotheses and study objectives

The hypotheses of this study are:

1. Acid suppressants will alter *in vitro* healthy (i.e. murine) and neoplastic (e.g. human, canine) mast cell intracellular structures in a time and concentration dependent fashion as determined by light and transmission electron microscopy. The proton pump inhibitor will have more pronounced treatment effects.
2. Only the proton pump inhibitor will cause significant cytotoxicity to neoplastic mast cells as assessed via validated flow cytometric (e.g. apoptosis, necrosis) and colorimetric (i.e. cytotoxicity) assays.
3. The proton pump inhibitor will have a pronounced treatment effect in inhibiting neoplastic mast cell function (e.g. degranulation) compared to famotidine.
4. All of these treatment effects will be diminished or absent when the *in vitro* neoplastic B cell lymphoma line (canine B cell 1771) is also treated with the same acid suppressants.

The objectives of this study are:

1. To evaluate the treatment effect of the acid suppressants, famotidine and esomeprazole, on the following outcome variables across multiple species:
    a. Healthy and neoplastic *in vitro* mast cell structure
    b. Neoplastic cell death
    c. The ability of mast cells to degranulate with different types of stimuli following acid suppressant treatment

1.9 References

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CHAPTER II

ESOMEPRAZOLE INDUCES CYTOTOXICITY AND STRUCTURAL CHANGES TO *IN VITRO* CANINE AND HUMAN NEOPLASTIC MAST CELLS

Objectives: Our primary study objective was to evaluate and compare the effects of esomeprazole, famotidine, and vehicle-treatment on mast cell (MC) ultrastructure, viability, and function *in vitro* using both healthy and neoplastic MCs.

Methods: Murine bone marrow derived (BMMC), human LAD2, and canine C2 and BR cells, were used for these studies, representing a single healthy (i.e., BMMCs) and multiple neoplastic MC models (i.e., LAD2, C2, BR), respectively. The rat basophilic leukemic (RBL-2H3) and canine B cell lymphoma 17-71 cell lines served as granulocytic and agranulocytic control lines for experiments, respectively. The treatment effect of acid suppressants on MC ultrastructure was assessed via both light (i.e., BMMC, BR and C2) and transmission electron microscopy (TEM; BMMC, C2, and LAD2). Differences in MC viability between groups was assessed via MTS-based, colorimetric assays and flow cytometry. Degranulation was assessed by quantification of β-hexosaminidase (i.e., LAD2 and RBL-2H3).

Results: Esomeprazole-treated MCs of all lines exhibited dramatic time and concentration-dependent alterations in ultrastructure (i.e., increased cytoplasmic vacuolization, compromise of cell membrane), increased apoptosis, and altered degranulation responses in comparison to famotidine and vehicle-treated cells. The canine B cell lymphoma cells consistently exhibited either no significant (i.e., cytotoxicity assays) or greatly diminished treatment responses (i.e., apoptosis) compared to MCs.

Significance: Esomeprazole, but not famotidine, induces significant cytotoxicity, as well as alterations to cell structure and function to multiple lines of *in vitro* neoplastic MCs. Continued *in vitro* work investigating the specific mechanisms by which PPIs induce these effects, as well as prospective, *in vivo* work comparing the treatment effects of acid suppressants on canine MCTs, are warranted.

2.1 Introduction

Acid suppressants (e.g. histamine-2 receptor antagonists [H_2RAs] and proton pump inhibitors [PPIs]) are considered first-line medical treatments for humans and dogs with gastric acid-related disorders. In comparison to H_2RAs, PPIs (e.g. omeprazole) are superior in their ability to raise intragastric pH in both humans and dogs, [1,2] and have now become the standard of care for the treatment of gastroduodenal ulceration and bleeding. [2] The reason behind the efficacy of PPIs compared to H2RAs is multifactorial, but predominantly hinges on their mechanism of action (MOA), which is to irreversibly block the final step of acid production by the parietal cell by inhibiting the hydrogen potassium ATPase (H+-K+-ATPase) pump. Common disorders in which acid suppressants are prescribed by veterinarians include GI ulceration and bleeding, esophagitis, and mast cell tumor (MCT) disease in dogs.

Mast cell tumors represent the most common cutaneous neoplasm in dogs worldwide, with up to an estimated prevalence of 8-20%. [3] While malignant mast cell (MC) disease in humans differs phenotypically from that in companion animals, there are many similarities in pathogenesis and behavior, including similar receptor expression and stimuli for MC degranulation. As MCs are integral first line sentinel cells of the immune system in response to an allergic or parasitic threat, they can be triggered to degranulate secondary to an immunoglobulin-E (IgE) mediated pathway via the high affinity Fc-epsilon receptor (FcƐR1) or

via a variety of other non-IgE immunologic stimuli (e.g. cytokines, substance P [SubP]). Mast cells contain a wide array of acidic, biogenic amines, and pro-inflammatory compounds that are released upon MC degranulation. With MCTs, degranulation can be especially detrimental due to the release of inflammatory mediators that might cause life-threatening, devastating adverse events (e.g. vasodilation, angioedema, GI ulceration, anaphylaxis).

Gastrointestinal ulceration induced by MCTs likely occurs secondary to excessive histamine release, which in turn stimulates release of hydrochloric acid (HCl) from gastric parietal cells with ensuing gastric hyperacidity. Thus, use some form of acid suppressant therapy in the medical management of MCT disease is a logical choice, whether this is pre-operatively for resectable tumors or as part of palliative care for metastatic disease. However, there are no studies in which acid suppressants have been shown to be beneficial in reducing adverse events in dogs with MCT disease.

In addition to their gastric acid suppressing properties, both H2RAs and PPIs exhibit anti-inflammatory and cytotoxic effects. The most relevant of these effects in regard to MCT disease are the ability of acid suppressants to decrease pro-inflammatory cytokines, exert cytotoxic effects on inflammatory and neoplastic cells, and even prevent neoplastic cell proliferation and metastasis. For example, omeprazole treatment alters *in vitro* murine bone marrow derived mast cells (BMMC) morphology and function. [4] Proton pump inhibitor treatment decreased MC degranulation and reduced histamine, pro-inflammatory cytokine (e.g. interleukins (ILs)- 4, 5, 13, and tumor necrosis factor alpha [TNF-α]) release from MCs. [4] The mechanism of action for the anti-inflammatory and cytotoxic effects of PPIs is unknown, however, one hypothesis is a direct effect on an ATPase pump called the vacuolar ATPase (V-ATPase). These pumps are found on the surface of both healthy and neoplastic cells and have a critical role in maintaining

an ideal intra and extra-cellular pH for cell homeostasis. Indeed, MCs depend on maintenance of an acidic environment for normal function and proliferation. [5,6] Moreover, while PPIs are not specific V-ATPase inhibitors, they act on many extra-gastric ATPase pumps. Blockade with specific V-ATPase inhibitors (i.e., bafilomycin A [BafA])[5] results in changes to MC (e.g., murine bone marrow-derived mast cell [BMMC]) structure and function *in vitro*, similar to what was seen with omeprazole. [4] There are no studies in which the effect of PPIs on canine neoplastic MCs have been evaluated. Moreover, the effects of PPIs other than omeprazole (e.g. esomeprazole) have not been widely evaluated. A comparative study aimed to evaluate the effects of acid suppressants on *in vitro* or *in vivo* MCs is necessary. If PPIs are found to have superior effects on canine MC viability and function, compared to H_2RAs, this data would serve as a rationale for *in vivo* clinical trials and would serve as a basis for the development of a consensus on the use of acid suppressants in dogs with MCT disease.

Our study objectives were to investigate and compare the treatment effects of an H_2RA (famotidine) or PPI (esomeprazole) on rodent, human, and canine *in vitro* neoplastic MC ultrastructure, viability and degranulation profiles. We hypothesized that esomeprazole would have more pronounced treatment effects compared to famotidine or vehicle-treated cells.

2.2 Materials and methods

Acid suppressants and vehicle control

Injectable famotidine (i.e., Pepcid®; Merck) and esomeprazole (i.e., Nexium®; Auromedics Pharmaceuticals) were purchased from the manufacturer via the pharmacies at the University of Tennessee College of Veterinary Medicine and Texas A&M University College of Veterinary Medicine & Biomedical Sciences. Both injectables were reconstituted with 0.9%

sodium chloride (regular saline; Hospira, Inc, Lake Forest, IL). Three different concentrations of famotidine and esomeprazole each were chosen based on the published literature, either investigating cytokine profiles in intestinal biopsies following famotidine treatment [7] or treatment of healthy Beagle dogs with clinically relevant doses of esomeprazole. [8,9] The lowest esomeprazole concentration was set to mimic that of peak plasma concentrations, [8,9] with the idea that higher concentrations might recapitulate PPI concentrations in the tissue of interest (i.e., intracellular concentrations). 0.9% saline was used as a vehicle control (VC) and applied at a volume equivalent to the largest volume of acid suppressant treatment (i.e., highest concentration PPI). Both acid suppressants and 0.9% saline were prepared fresh and mixed into the cell culture media at the time of each experiment.

Cell culture

All *in vitro* cell lines were maintained in either T25, T75 or T150 tissue culture flasks (Falcon® or VWR®).

Murine bone marrow-derived mast cells (BMMCs) were generated as previously described,[10] with collection of cells from the femurs of mice performed immediately postmortem. Cells were then cultured in RPMI 1640 media (Corning®) with fetal bovine serum (FBS) (Gibco™), penicillin (100 U/ml)/streptomycin (100 µg/ml) (Corning®), sodium pyruvate (Corning®), HEPES, and MEM nonessential amino acids (Corning®) in the presence of interleukin-3 (IL-3) and stem cell factor (SCF), both at 5 ng/ml and purchased from R&D Systems® (Minneapolis, MN). Cells were cultured for a minimum of 8 weeks prior to use in assays, with media changed weekly and sub-culturing performed weekly. Purity of MCs was assessed via toluidine blue staining and confirmed by flow cytometric assessment for c-kit (i.e.,

CD117+) and FcƐR1 receptors prior to use in experiments. Cells <90% double positive for both receptors were excluded.

Human LAD2 cells,[11] originally harvested from a man with mast cell leukemia, were acquired from the National Institutes of Health (NIH; Bethesda, MD) and maintained in StemPro™-34 with Nutrient Supplement (Gibco™), l-glutamine (2 mM) (Gibco™), penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco™), and recombinant human stem cell factor (rhSCF) (R&D Systems®) at 100 ng/ml. Cells were cultured for a minimum of 6 weeks prior to use, and media was hemi-depleted with fresh rhSCF, which was replenished weekly. LAD2 cultures not allowed to grow past 500,000 cells/mL, and were passaged every 1-3 weeks depending on growth

The canine C2 and BR mastocytoma cell lines were harvested from different dogs with MCTs. [12,13] The BR line is non-adherent, while the C2 line has characteristics of both adherent and non-adherent cell lines. Both cell lines were maintained in Dulbecco's Modified Essential Medium (DMEM) with penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco™), 2 mM glutamine (Gibco™), and fetal bovine serum (FBS) (Gibco™). The media was changed twice weekly, and cells sub-cultured weekly or bi-weekly depending on cell density.

Rat basophilic leukemic (RBL-2H3) cells, originally harvested from rats with a chemically induced basophilic leukemia, were obtained from the NIH. This line represented another granulocytic cell line well validated for the study of MC activation, given that they also contain the high affinity IgE surface receptor (i.e., FcƐR1). [14] They were maintained in Eagle's Minimum Essential Medium (MEM) (Corning™) with penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco™) and fetal bovine serum (FBS) (Gibco™). Media was changed twice weekly, and cells were sub-cultured weekly.

To investigate the specificity of treatment effects towards granulocytic (e.g., MCs and RBL-2H3) lines, experiments were also performed using an agranulocytic, neoplastic *in vitro* canine cell line (B cell lymphoma 1771). The 17-71 cell line, an adherent cell line, was originally obtained from a dog with B cell lymphoma. [15]

For all experiments, cell cultures were either deprived of FBS or rhSCF (LAD2 only) in order to prevent replication of cells during the assay. Cells passaged more than a total of 60 times in their lifetime were not used in experiments, as were those passaged no more than 20 times out of cryopreservation in liquid nitrogen (N_2). The least differentiated MC line in our experiments (BR) was found to retain comparable histamine contents up to at least 20 passages out of cryopreservation, [13] which led to establishment of this criteria. The LAD2, C2, BR, and 17-71 lines were screened for mycoplasma infections routinely (e.g. at least every 3 months) with a validated kit for detection of bacterial contamination (MycoAlert™ Mycoplasma detection kit, Lonza Group, Switzerland). Positive results were read via quantification of luminescence according to manufacturer instructions (Gen5 Analysis Software, BioTek® Synergy 2 plate reader).

Measurement of pH in treated cell culture media

To account for any differences in outcome variables secondary to significant differences in the pH of H2RA or PPI treated cell culture media, the pH was measured for all treatment groups before and after incubation in a 37º C, 5% CO_2 atmosphere (Orion Star A211 pH Meter, ThermoFisher Scientific, Waltham, MA, USA).

Light and transmission electron microscopy (TEM)

Mast cell lines used for light microscopy evaluation included the BMMC, BR, and C2 lines, and those used for transmission electron microscopy (TEM) included the BMMC, BR, C2, and LAD2 lines. Cells were seeded at a concentration of 200,000 cells/mL into either T25 flasks (BMMC, BR) or onto coverslips in 6 well plates 2-3 days prior to use in ultrastructure assays. Serum free media was used in order to prevent replication of cells during the duration of the assay. Each flask was treated with either VC, and either three different concentrations of famotidine (3 ug/mL [0.01 µM], 33 ug/mL [0.1 µM], or 330 ug/mL [1 µM]), esomeprazole (9 µg/mL [0.03 µM], 90 µg/mL [0.3 µM], or 900 µg/mL [3 µM]), or no treatment at all (i.e., untreated media). Every 12 hours, fresh media with treatment was reapplied to each flask. An equivalent of 500,000 cells was removed from each flask at four total time points (e.g. 0, 6, 24, and 48 hours) for either light or transmission electron microscopy (TEM). An equivalent amount of fresh, non-serum treated media was used to replace the volume removed at each time point.

For light microscopy, a total volume of 200 µL maximum (i.e., 100,000 cells per slide/sample) per sample was applied to glass slides via a cytospin (Cytospin 4, ThermoFisher Scientific, Waltham, MA, USA) at 1000 rpm x 5 min and then stained with Wright's stain. An Olympus DP73 (model BX43F) microscope was used to capture images of MCs at 40x or 100x magnification for all cells.

Non-adherent MC samples undergoing TEM (BMMC, LAD2) were fixed in either a 2-2.5% glutaraldehyde 0.1 M cacodylate (Glut/CaCo)-HCl buffer (pH 7.2-7.3) fixative (BMMC) or suspended in an agarose gel matrix in a 96 well plate (LAD2) prior to sectioning and imaging. Murine bone marrow mast cells were centrifuged at 1500 rpm for 5 minutes and the cell pellet (1,000,000 cells per sample) resuspended in the Glut/CaCo fixative prior to processing at the University of Georgia Electron Microscopy Center (UGA). LAD2 cells were plated at a

concentration of 100,000 cells/well in 96 well plates, and allowed to incubate in media without rhSCF for 24 hours prior to application of treatments. At each time point, plates were centrifuged at 12,000 rpm (400 x g) for 5 minutes, then resuspended in a 1:1 2% Glut/CaCo 1% agarose gel to a total volume of 200 µL per well. Samples were kept refrigerated until all time points were completed, then delivered to the Texas A&M Microscopy and Imaging Center (TAMU).

Processing of all cells for TEM included fixation in a 2% Glut/CaCo buffer followed by incubation with either a 1% osmium tetroxide/0.1M CaCo-HCl (UGA) or 1% OsO_4 / 1% $K_4[Fe(CN)_6]$ /0.1M CaCo-Hcl (TAMU) buffer for 30 minutes. All samples were then washed for 10 minutes with water on a shaker at RT, and TAMU samples were incubated with 0.4% uranyl acetate for 20 minutes on a shaker at RT. All samples were then dehydrated in an ethanol series (i.e., 30%, 50%, 75%, 95%, 100%). Cells were treated with propylene oxide (PO) or ethanol prior to infiltration with an Epon-Araldite or Epon-812 mixture prior to embedding. The UGA samples were prepared for imaging using a Reichert Ultracut S ultramicrotome (Leica, Inc., Deerfield, IL) with post staining performed with an aqueous uranyl acetate and Reynolds Lead Citrate. A JEOL JEM-1011 Transmission Electron Microscope (JEOL USA, Inc., Peabody, MA) was used to view grids prior to image acquisition on an AMT XR80M Wide-Angle Multi-Discipline Mid-Mount CCD Camera (Advanced Microscopy Techniques, Woburn, MA). Images were acquired at magnifications ranging from 2,000 to 2,500X. At TAMU, all tissues were sectioned ultra-thin (~ 100 nm thickness) with a Leica UC6 ultramicrotome and Diamtome diamond knife and placed on a Cu grid. A FEI Morgagni 268 transmission electron microscope, equipped with an ImageView III CCD camera, was used to collect TEM images for C2 and LAD2 cells. No post-staining was performed on C2 or LAD2 cells (TAMU). Images were acquired at magnifications ranging from 11,000 to 56,000X.

Quantification of cytoplasmic vacuoles and mitochondria (TEM)

Image J software (ImageJ, Fiji, NIH) was used to quantify some of the qualitative changes noted in TEM images (e.g., increased cytoplasmic vacuolization) and assess for any differences in the size of the mitochondria across treatment groups. Vacuole and mitochondria measurements were normalized to the cytoplasm area for each cell. Per treatment group per timepoint (i.e., baseline, 24, and 48 hours), 5 cells were quantified. These represented 5 replicates per treatment group. Electron microscopy images were opened in Adobe Photoshop, and the number of pixels used to accurately scale the size of the cell and cytoplasmic structures for measurements in ImageJ. Per cell, four cross sectional measurements of each cytoplasmic vacuole were acquired in ImageJ, and the medians of these measurements used to represent the size of one vacuole. Mitochondrial width was measured for each Mito per cell, and the median of these measurements used to represent the Mito width per cell. In order to quantify the cytoplasm area per cell, the total area of each cell and the cell nucleus was quantified (ImageJ), and the nucleus area subtracted from the total cell area to quantify the cytoplasm area. The sum of the vacuole medians per cell was then divided by the cytoplasm area in order to generate a total vacuole size per cytoplasm area ratio (total vacuole size: cytoplasm area) for each of the 5 cells (i.e., 5 replicates) within a treatment group. Similarly, the median mitochondria width per cell was divided by the cytoplasm area in order to generate a mitochondria width per cytoplasm area ratio (mitochondria width: cytoplasm area).

Normality was assessed via a Shapiro-Wilk test, and differences between groups assessed with a One-Way ANOVA and Dunnett's post hoc (GraphPad Prism 9.0.2 Software, San Diego, CA).

Cytotoxicity assays

Cytotoxicity was quantified using a commercially validated assay (MTS Cell Title 96® Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI) according to the manufacturer's instructions. Cells were seeded into 96 well plates at a density consistent with 300,000 total per well, with a minimum of 3 replicates per treatment group. Cells were allowed to proliferate in media lacking any treatments for 12 hours, then VC or acid suppressant treatment was applied. After a 12-hour incubation with treatment, all media was changed to regular media prior to addition of 20 µl/well of the MTS Promega solution and incubation at 37◦C at 5% CO_2 for 1 hour prior to analysis. Cell viability was measured as the optical density read at a wavelength of 490 nm (OD_{490nm}) with a BioTek® Synergy 2 plate reader and Gen5 Analysis Software. Normality was assessed via a Shapiro-Wilk test, and differences between groups assessed with a One-Way ANOVA and Dunnett's post hoc tests (GraphPad Prism 9.0.2 Software, San Diego, CA).

Apoptosis assays (early and late apoptosis)

Fluorescent probes were either purchased from Invitrogen™ Molecular Probes™ (Annexin V [FITC-conjugated fluorophore]; ThermoFisher Scientific, Waltham, MA, USA) or BD Biosciences (Annexin V [FITC-conjugated fluorophore], propidium iodide [PI; Texas Red-conjugated fluorophore]; ThermoFisher Scientific, MA, USA) and used to denote early or late apoptosis via flow cytometry. Compensation, using chemically induced apoptotic cells (i.e., 12-hour incubation with 5 mM H_2O_2 control[16]) single stained with either Annexin-V- or PI, along with live, unstained controls, was performed in order to account for overlap of fluorescent intensity between the FITC and Texas Red channels. 10% hydrogen peroxide (H_2O_2) used as a positive control for apoptosis,[16] with Annexin V^+/PI^- and Annexin V^+/PI^+ positive cells used to denote either early or late apoptosis, respectively. All treated cells were normalized to the

untreated control group, so that baseline apoptosis from untreated controls were not included in gating. Non-adherent (e.g., LAD2, BR) and adherent (e.g., C2, B cell 1771) cells were treated with VC or acid suppressants as described in the above methods. Following incubation with respective treatments, cells were washed twice with cold 1x DPBS. The C2 and 17-71 lymphoma cell lines were trypsinized following incubation with their respective treatments, following which a soybean trypsin inhibitor kit (Gibco™, ThermoFisher Scientific, Waltham, MA, USA) was used to prevent trypsin-induced cell death, given the absence of serum. Cells were suspended to a concentration of 1 million cells/sample in 1x Annexin V binding buffer (BD Biosciences kit) as a fixative, and the cell pellet incubated with 5 µl each of Annexin and PI stain for 15 minutes in the dark on ice prior to analysis. Anywhere from 500-1,000 µl of flow or binding buffer was added to each sample, and the sample vortexed, prior to analysis. Samples were analyzed on a BD Fortessa X-20 (TAMU COM-CAF facility). A minimum of 10,000 and maximum of 30,000 events were captured per sample. Experiments were performed in triplicate.

Degranulation

Beta-hexosaminidase (β-hexosaminidase) assays were performed in order to quantify changes in degranulation between treatment groups for the LAD2 and RBL-2H3 cells.[14,17] The canine B cell lymphoma 17-71 line was used as an agranulocytic, negative control. Cells were seeded into 96 well plates at a density of 50,000 cells in a total volume of 300 µL per well. LAD2 cells were suspended in StemPro media deprived of rhSCF, and allowed to incubate for 24 hours prior to treatment with acid suppressants or controls. Treatments were applied and allowed to incubate for 12 hours prior to stimulation with either calcium ionophore A23187 or substance P (SubP). Altered MC function secondary to PPI treatment has been documented as soon as 2 hours post treatment,[4] justifying this incubation period. The A23187 represents

chemical induction of degranulation, and does not recapitulate an *in vivo* pathway for degranulation, as does the SubP. Control groups included an unstimulated control, untreated positive control (i.e., no treatment but degranulation stimulated), and VC.

Following incubation with respective treatments, 96 well plates were centrifuged at 400 x g for 5 minutes and cells washed twice with Tyrode's buffer (containing sodium chloride, potassium chloride, calcium chloride, magnesium chloride, glucose, bovine albumin fraction V, and 1 M Hepes in distilled water, pH 7.4). Cells were then resuspended in 100 µl of either 10 µM A23187 or 10 µM SubP and allowed to incubate for either 2 or 8 hours, respectively, to stimulate degranulation. Following stimulation, plates were again centrifuged at 400 x g for 5 minutes and 30 µL of the supernatants and the cell pellets, respectively, were individually combined with 10 µL of p-nitrophenyl-N-acetyl-β-D-glucosaminide (NAG). The NAG acts as a substrate for β-hexosaminidase, and will precipitate, leading to a yellow quantifiable color change via measurement of the optical density read at a 405 nm wavelength (OD_{405}). Following aspiration of 30 µL supernatant from each well, as much of the remaining supernatant was removed and 30 µL of LAD2 cell pellet was obtained via gentle pipetting to mix cells with the remaining Tyrode's buffer. Because the 17-71 line is adherent, 0.1% Triton-100X in Tyrode's buffer was used as a detergent to gently disrupt the adherent cell pellets prior to combination with the NAG. Plates were incubated at 37% in 5% CO_2 for 1 hour prior to the addition of 100 µl carbonate buffer (sodium bicarbonate, sodium carbonate, pH 10) to stop the reaction and precipitate a color change for positive results. Optical densities were read with a BioTek® Synergy 2 plate reader and Gen5 Analysis Software. Three replicates were performed per treatment group, with a minimum of 3 experiments performed for each cell line.

The percentage (%) of degranulation (i.e., % β-hexosaminidase release) was calculated as follows: 100 x ([OD supernatant]/[OD supernatant + OD cell pellet] – OD blank Tyrodes buffer only well). Experiments with baseline, unstimulated controls with % degranulation higher than 20% were excluded.

The canine C2 and BR cell lines were unreliable models for degranulation, with minimal color change despite addition of degranulation stimuli, indicative of negative β-hexosaminidase release.

Normality was assessed via a Shapiro-Wilk test, and differences between groups assessed with a One-Way ANOVA and Dunnett's post hoc (GraphPad Prism 9.0.2 Software, San Diego, CA).

2.3 Results

Measurement of pH in treated cell culture media

No significant differences were found in the pH of acid suppressant treated cell culture media versus VC or untreated control cells.

Light and transmission electron microscopy (TEM)

Light microscopy images were qualitatively assessed for differences in cell size, granule size, and morphology, and other general morphologic changes between treatment groups for the BMMC, BR, and C2 cell lines. Images were acquired at baseline, 24, and 48 hours after treatment. Subjectively, there were concentration- and time-dependent changes in cell size, number, and morphology (i.e., shrinkage of cells, reduction in cell number) in the esomeprazole

treated cells compared to famotidine treated and VC groups for all MCs imaged. See **Figures 2.1-2.2**.

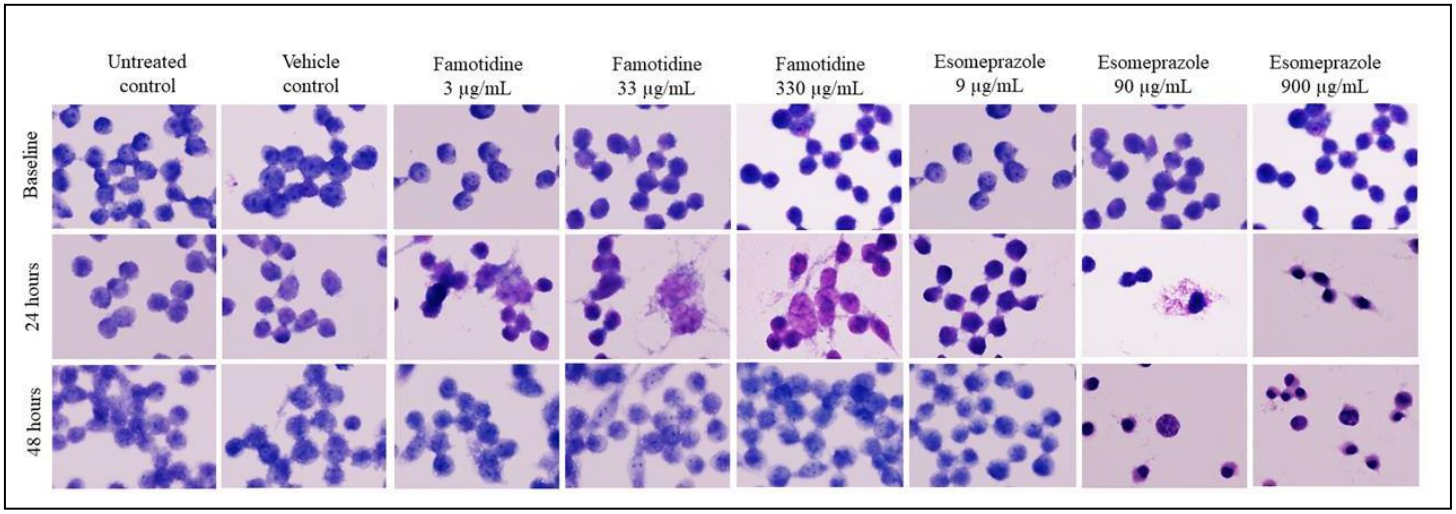

Figure 2.1. Esomeprazole, but not famotidine treatment, causes qualitative decreases in murine MC density and loss of an intact cell membrane. This figure shows murine BMMCs (P34), treated with either vehicle control, or increasing concentrations of famotidine or esomeprazole for 24 or 48 hours. Note the relative lack of visible changes with famotidine treatment, even after 48 hours, in contrast to the changes in MC morphology (e.g. loss of clear cell membrane) and number even after 24 hours with esomeprazole treatment. Scale bar set at 10 µm, images stained with Wright-Giemsa stain, and acquired at 100X magnification.

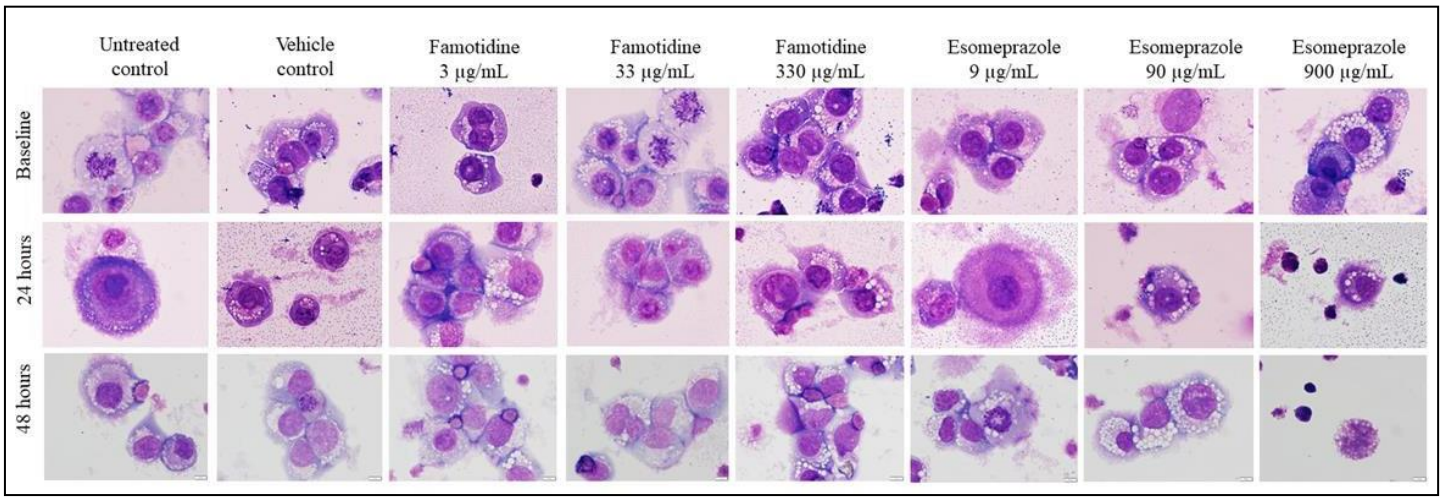

Figure 2.2 Esomeprazole, but not famotidine, causes concentration- and time-dependent treatment effects to neoplastic canine MCs. Non-adherent, canine neoplastic BR MCs (P14), treated with either vehicle control, or increasing concentrations of famotidine or esomeprazole

for 24 or 48 hours. Note the relative lack of visible changes after famotidine treatment, even after 48 hours; in contrast to the changes in MC morphology and number even after 24 hours with esomeprazole treatment. Scale bar set at 10 µm, images stained with Wright-Giemsa stain, and acquired at 100X magnification.

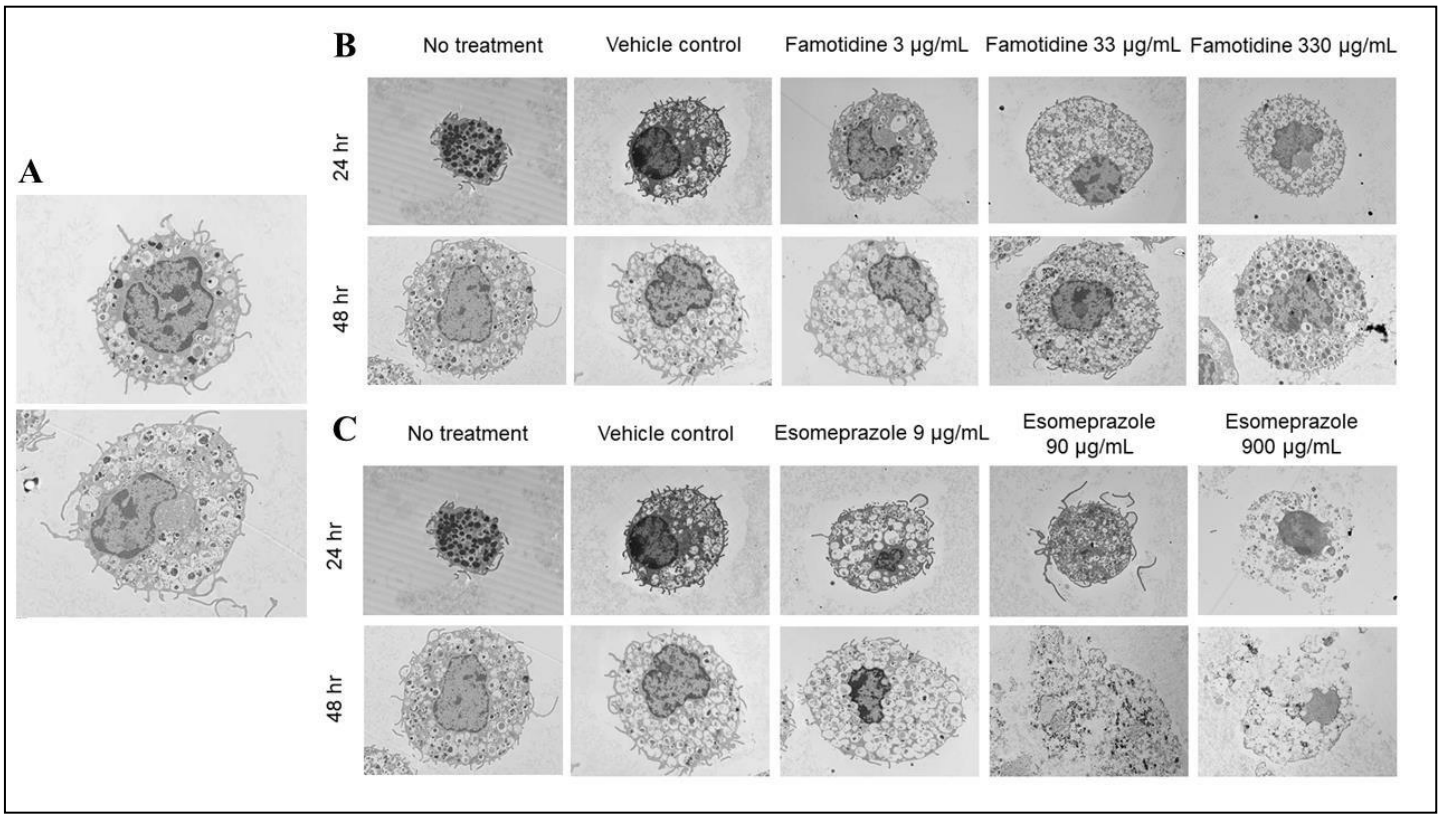

Figure 2.3. Murine BMMCs at either baseline (A; top= untreated control, bottom= VC), 24, or 48 hours following control, famotidine (B) or esomeprazole (C). Note disruption to the nucleus as well as intact cell membrane in esomeprazole-treated groups. Images acquired at 2,000 to 2,500X magnification (University of Georgia College of Vet Med).

Transmission electron microscopy (TEM) images were acquired at baseline, 24, and 48 hours for the BMMC and canine C2 lines. Concentration- and time-dependent qualitative differences in cell size and number, as well as an increase in cytoplasmic vacuolization, were noted in the esomeprazole treated groups for all MCs in comparison to famotidine and VC treated cells. See **Figures 2.3-2.6**. There was a significant increase in total vacuolar size per cytoplasmic area (total vacuolar size: cytoplasm area ratio) of canine C2 MCs treated with

esomeprazole in comparison to famotidine or VC at both 24 and 48 hours (**Figure 2.5**). No significant differences in mitochondrial size per cytoplasmic area were found for any treatment group at any time point (**Figure 2.6**).

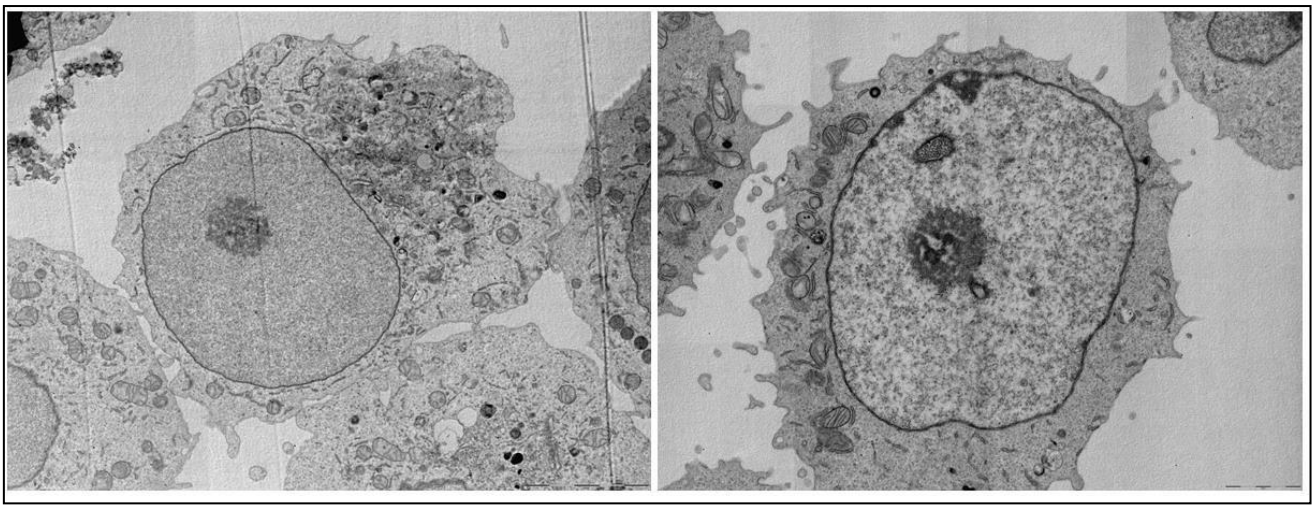

Figure 2.4. Canine neoplastic C2 MCs (P8), treated with either DMEM media (untreated; left), or vehicle control (VC; right) at baseline. All baseline (T0) images for famotidine and esomeprazole-treated cells were comparable to untreated and VC. Note the clear delineation between nucleus and cytoplasm, the visible mitochondria, and intact cell membrane. Images acquired at 11,000 to 56,000X magnification, scale bar at 2 or 5 µm (TX A&M College of Vet Med, Image Analysis Center).

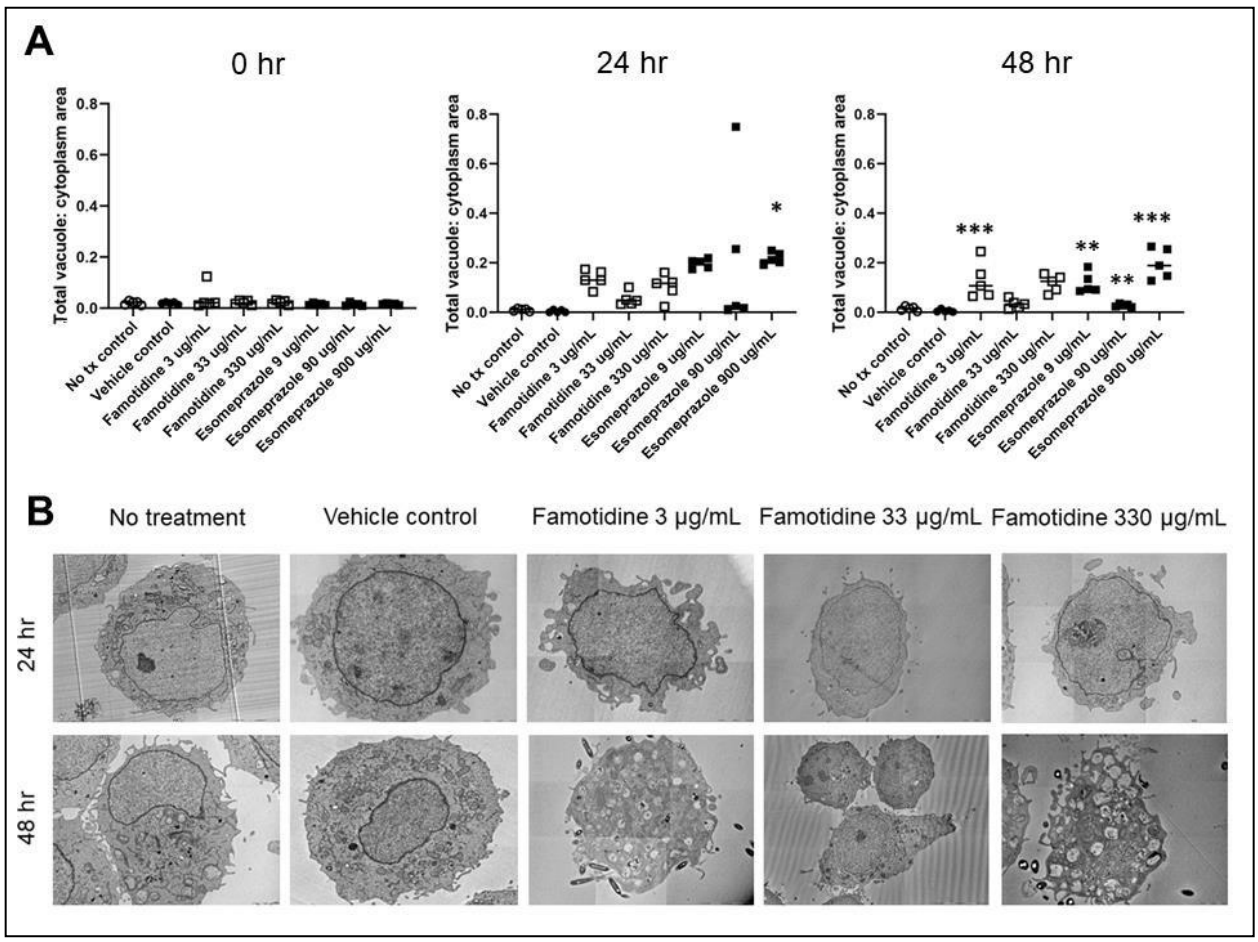

Figure 2.5. Famotidine treatment causes some visible structural changes of canine neoplastic MCs after 48 hours, but only esomeprazole treatment consistently changes the total cell vacuole: cytoplasm ratios. Mean total vacuole: cytoplasm ratios at 0, 24, and 48 hours (A) and TEM images at 24 and 48 (B) hours for canine C2 MCs (P8) treated with control or escalating concentrations of famotidine. A significant increase in mean total vacuole: cytoplasm ratio is seen for all esomeprazole-treated cells in comparison to untreated and vehicle control (B). Note the visible increase in vacuolization for some of the famotidine-treated cells at 48 hours (B). Normality assessed via Shapiro-Wilk, One-Way ANOVA used to detect differences between groups (GraphPad Prism 9.0.2, San Diego, CA). * indicate statistical difference to baseline (no tx control) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Images acquired at 11,000 to 56,000X magnification, scale bar at 2, 5 or 10 µm (TX A&M College of Vet Med, Image Analysis Center).

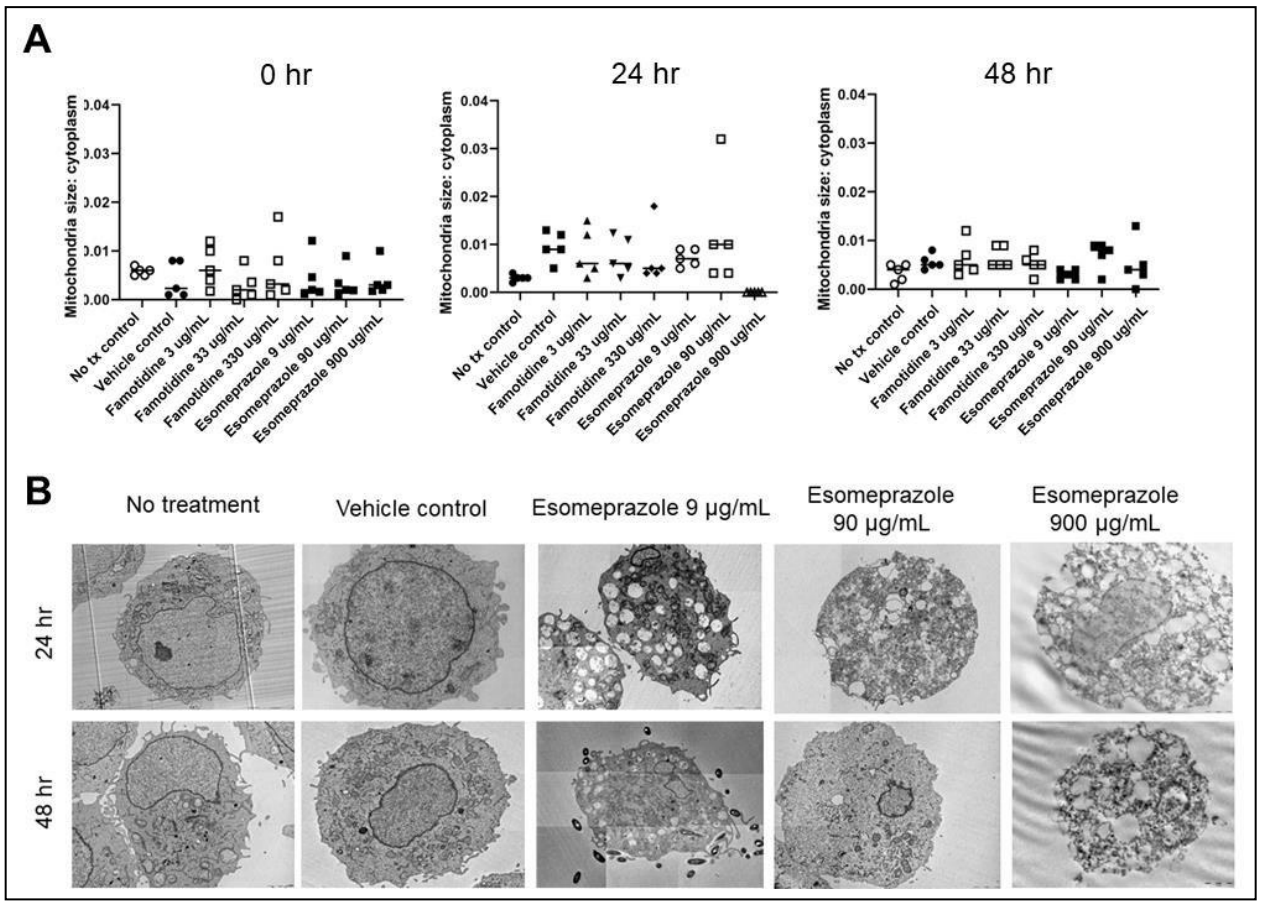

Figure 2.6. Esomeprazole treatment causes visible structural changes of canine neoplastic MCs after 24 and 48 hours, but neither famotidine nor esomeprazole changes the mitochondria: cytoplasm ratios. Mean total mitochondria : cytoplasm ratios at 0, 24, and 48 hours (A), and TEM images at 24 and 48 (B) hours for canine C2 MCs (P8) treated with control or escalating concentrations of esomeprazole. No significant increases were seen in the mean total mitochondrial: cytoplasm ratios for any acid suppressant treated groups in comparison to controls (A). Note the dramatic increase in vacuolization for esomeprazole-treated cells at both 24 and 48 hours (B). Normality assessed via Shapiro-Wilk, One-Way ANOVA used to detect differences between groups (GraphPad Prism 9.0.2, San Diego, CA). Images acquired at 11,000 to 56,000X magnification (TX A&M College of Vet Med, Image Analysis Center).

Cytotoxicity assays

A concentration-dependent, significant increase in MC death indicative of cytotoxicity was seen after esomeprazole treatment in the neoplastic human LAD2, canine C2, and canine BR cell lines after 12 hours, as well as RBL-2H3 cells (**Figure 2.7**). Canine B cell lymphoma 17-71 cells showed less cytotoxicity in response to esomeprazole treatment (**Figure 2.8**).

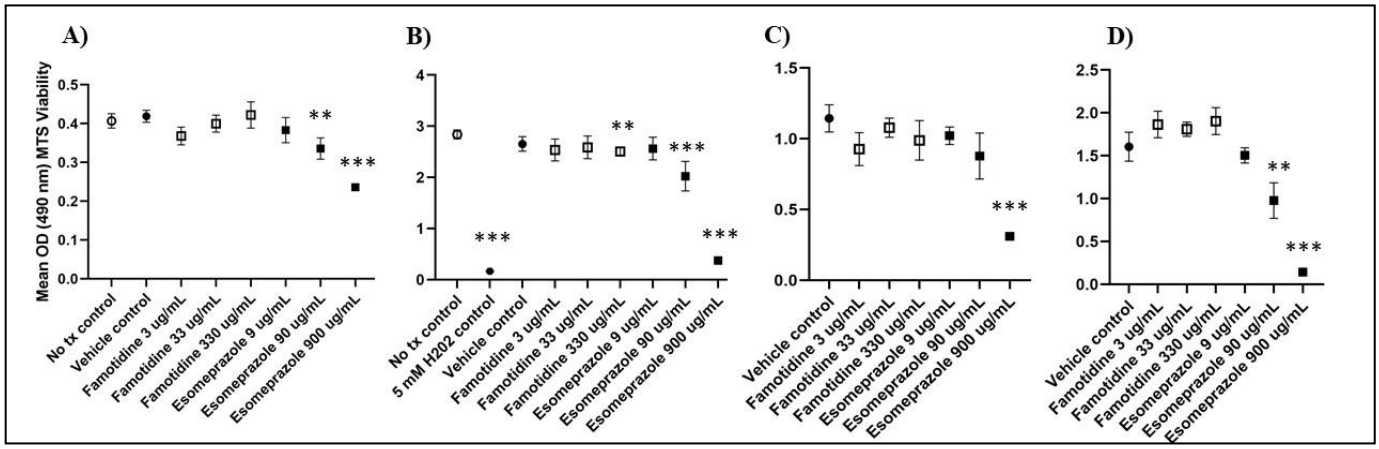

Figure 2.7. Esomeprazole causes cytotoxicity in a concentration-dependent manner to canine BR P9 (A), C2 P8 (B), and human LAD2 P20 (C) MCs, as well as rat RBL-2H3 P18 (D) cells, compared to both no treatment and vehicle controls after 24 hours of treatment. Normality assessed via Shapiro-Wilk, One-Way ANOVA used to detect differences between groups (GraphPad Prism 9.0.2, San Diego, CA). Mean ± standard deviation of three (n=3) replicates per treatment group. ** $P < 0.01$, *** $P < 0.001$. Experiment performed in triplicate (BR P8-9, C2 P8-11, LAD2 P20, 22 and 32).

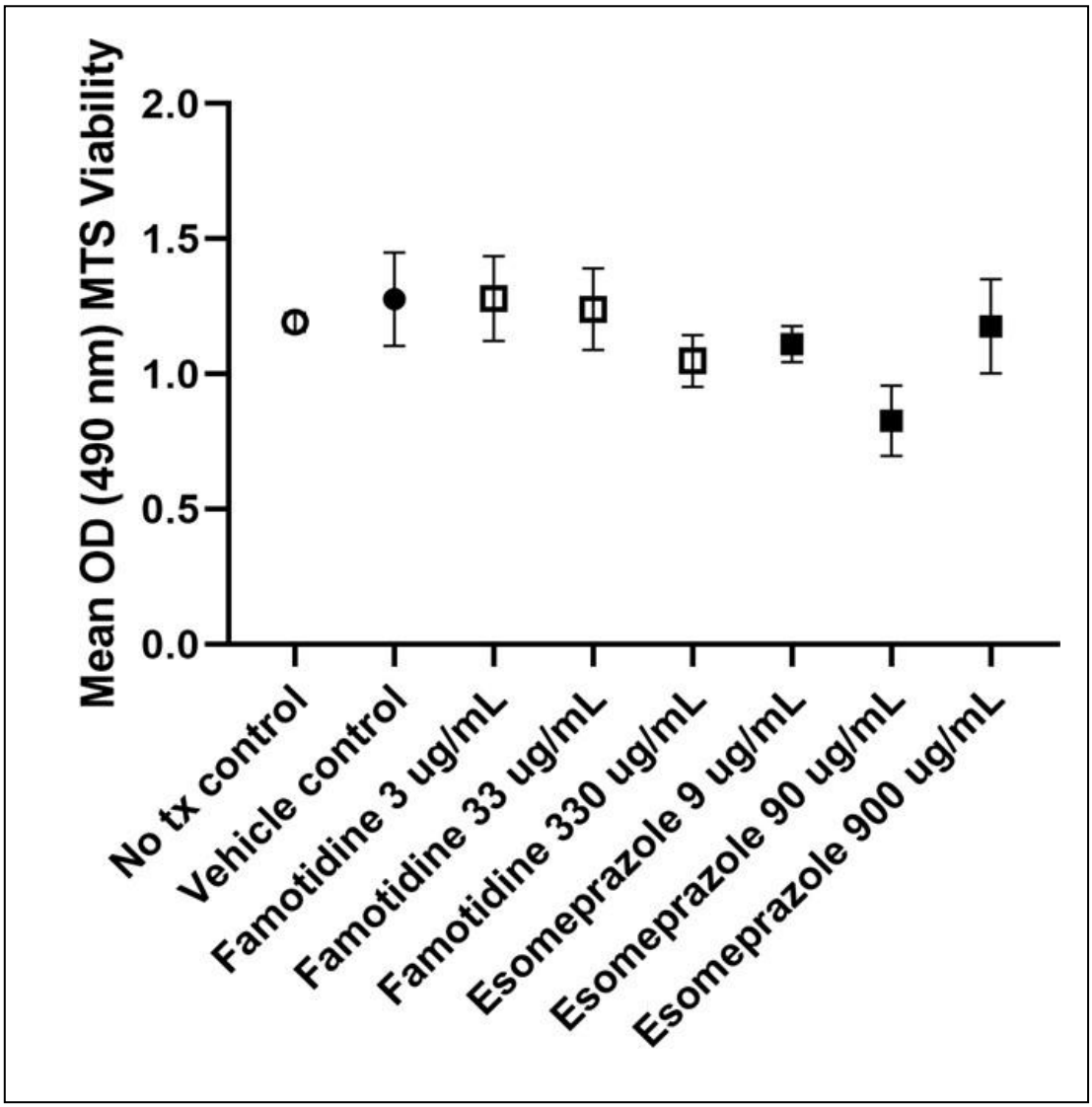

Figure 2.8. **Agranulocytic, lymphoma cells are less susceptible to the cytotoxic effects of esomeprazole compared to mast cells and basophils.** Neither famotidine nor esomeprazole caused significant cytotoxicity towards canine B cell lymphoma 17-71 cells (P8) after 24 hours of treatment. Normality assessed via Shapiro-Wilk, One-Way ANOVA used to detect differences between groups (GraphPad Prism 9.0.2, San Diego, CA). Mean $\pm$ standard deviation of three (n=3) replicates per treatment group. Experiment performed in triplicate (P8-9).

Apoptosis assays

Apoptosis was not evaluated beyond a 24-hour treatment period given that significant cytotoxicity was already seen following 12 hours of treatment in all MC lines evaluated. Relative increases in the percentage of cells in either early or late apoptosis was seen after esomeprazole

treatment of non-adherent BR and LAD2, and adherent C2 for all 3 experiments performed. Canine lymphoma 17-71 cells also showed a significant, but less severe, magnitude of apoptotic cells after 12 or 24 hours of treatment with esomeprazole. **Table 1** shows the median and range of the percentage of cells in early and late apoptosis for 12 and 24 hours for triplicate experiments. **Figure 2.9** shows differences in percentages of cells in early and late apoptosis following treatment with acid suppressants, VC, or no treatment control. **Figures 2.10 and 2.11** show differences in Annexin V and PI fluorescent intensities, respectively, for all cell lines following 12- or 24-hour treatments. The Y axes of all histograms were normalized to reflect relative intensities relative to the number of events acquired per sample (e.g. 10-30,000 events) (FlowJo™ Version 10 Software, BD Biosciences).

		12 hour treatment		24 hour treatment	
Treatment	**Type of cell**	**Early apoptosis Median % (range)**	**Late apoptosis Median % (range)**	**Early apoptosis Median % (range)**	**Late apoptosis Median % (range)**
Vehicle Control	Canine BR	**0.9** (0-1.8)	**4.6** (0-9.2)	**10.8** (0-23)	**56** (0-63.4)
	Canine C2	**0** (0-1.47)	**2** (0-2.2)	**0** (0-0)	**0** (0-0)
	Human LAD2	**0** (0-0)	**0.4** (0-0.7)	**0** (0-0.3)	**0.9** (0-2.1)
	Canine B cell lymphoma 17-71	**0** (0-0)	**0** (0-0)	**2.1** (0.1-4)	**1.5** (0-3)
5 mM H2O2 positive control	Canine BR	**0** (0-0)	**88.5** (83-93.9)	**0** (0-66)	**83** (14-94)
	Canine C2	**0** (0-0)	**90.1** (99.8-91)	**0** (0-0)	**89.5** (89.1-91.7)
	Human LAD2	**0.1** (0-0.1)	**90.8** (90-94.6)	**0.1** (0.1-0.7)	**91.2** (90.6-95.4)
	Canine B cell lymphoma 17-71	**3.55** (0-7.1)	**83.25** (79.5-87)	**0.15** (0-0.3)	**83.85** (76.4-91.3)
Famotidine 3 ug/mL	Canine BR	**1.1** (0-2.2)	**4.85** (0-97)	**2.4** (0.4-27.2)	**15.7** (0-58)
	Canine C2	**0** (0-0.12)	**0** (0-0)	**0** (0-0)	**1.4** (0-1.4)
	Human LAD2	**0** (0-0)	**0** (0-2.2)	**0** (0-0)	**0.9** (0-2.1)
	Canine B cell lymphoma 17-71	**1.8** (0-3.5)	**3.2** (3.2-3.2)	**0.5** (0-0.9)	**1.4** (0-2.7)
Famotidine 33 ug/mL	Canine BR	**0.95** (0-1.9)	**4.35** (0-8.7)	**2.2** (0-27)	**14.4** (1.3-57.9)
	Canine C2	**0.1** (0-0.7)	**0** (0-0.15)	**0** (0-0)	**0** (0-0)
	Human LAD2	**0** (0-0)	**0** (0-2)	**0.1** (0-0.2)	**0** (0-1.7)
	Canine B cell lymphoma 17-71	**1.9** (0.4-3.3)	**7.8** (3-12.6)	**0.95** (0.9-1)	**1.5** (0.3-2.6)
Famotidine 330 ug/mL	Canine BR	**1** (0-2)	**4.5** (0-8.9)	**1.7** (0-25)	**13** (0-58.2)
	Canine C2	**0** (0-0)	**0** (0-0)	**0** (0-0)	**0** (0-0)
	Human LAD2	**0** (0-0)	**0.5** (0-0.9)	**0.4** (0-0.6)	**0** (0-0.6)
	Canine B cell lymphoma 17-71	**2** (0-4)	**1.7** (0-3.3)	**1.4** (1.2-1.5)	**4.9** (0.7-9)
Esomeprazole 9 ug/mL	Canine BR	**1.3** (0-2.6)	**4.3** (0-8.4)	**1.8** (0-26.4)	**15.8** (0-57.7)
	Canine C2	**0** (0-0.67)	**0** (0-0.2)	**0** (0-0)	**0** (0-0)
	Human LAD2	**0** (0-0)	**1.1** (0-2.1)	**0.2** (0-0.3)	**0** (0-1.4)
	Canine B cell lymphoma 17-71	**1.7** (0-3.3)	**2.2** (0-4.4)	**1.9** (0.9-2.9)	**2.1** (1.4-2.8)
Esomeprazole 90 ug/mL	Canine BR	**16.7** (9-23.1)	**18.9** (6.5-30.2)	**5.8** (1.3-10.5)	**83.9** (72-85.3)
	Canine C2	**1.2** (0.1-1.44)	**1.9** (1.33-2.44)	**0** (0-0)	**0** (0-0)
	Human LAD2	**0** (0-0)	**0** (0-1.9)	**0.2** (0-0.3)	**0** (0-1.4)
	Canine B cell lymphoma 17-71	**2.3** (0-4.5)	**13.2** (6.5-20.1)	**2.3** (0-4.6)	**16.6** (12.1-21.1)
Esomeprazole 900 ug/mL	Canine BR	**0** (0-0)	**91.7** (84-99)	**0** (0-0.8)	**88.3** (79.9-98.8)
	Canine C2	**0** (0-0.7)	**92.1** (90.1-93.1)	**0** (0-0)	**85.2** (83.4-87.8)
	Human LAD2	**0** (0-0)	**64** (12.7-91.3)	**0** (0-0.1)	**88.6** (27.8-88.7)
	Canine B cell lymphoma 17-71	**0.15** (0-0.3)	**12.5** (0.2-24.7)	**7.8** (0-15.6)	**23.7** (8-39.4)

Table 2.1 Summary of early and late apoptosis for all cell lines at 12 and 24 hours, for both adherent (canine C2, B cell lymphoma 17-71) and non-adherent (canine BR, human LAD2) cell lines. Data is representative of each experiment performed in triplicate.

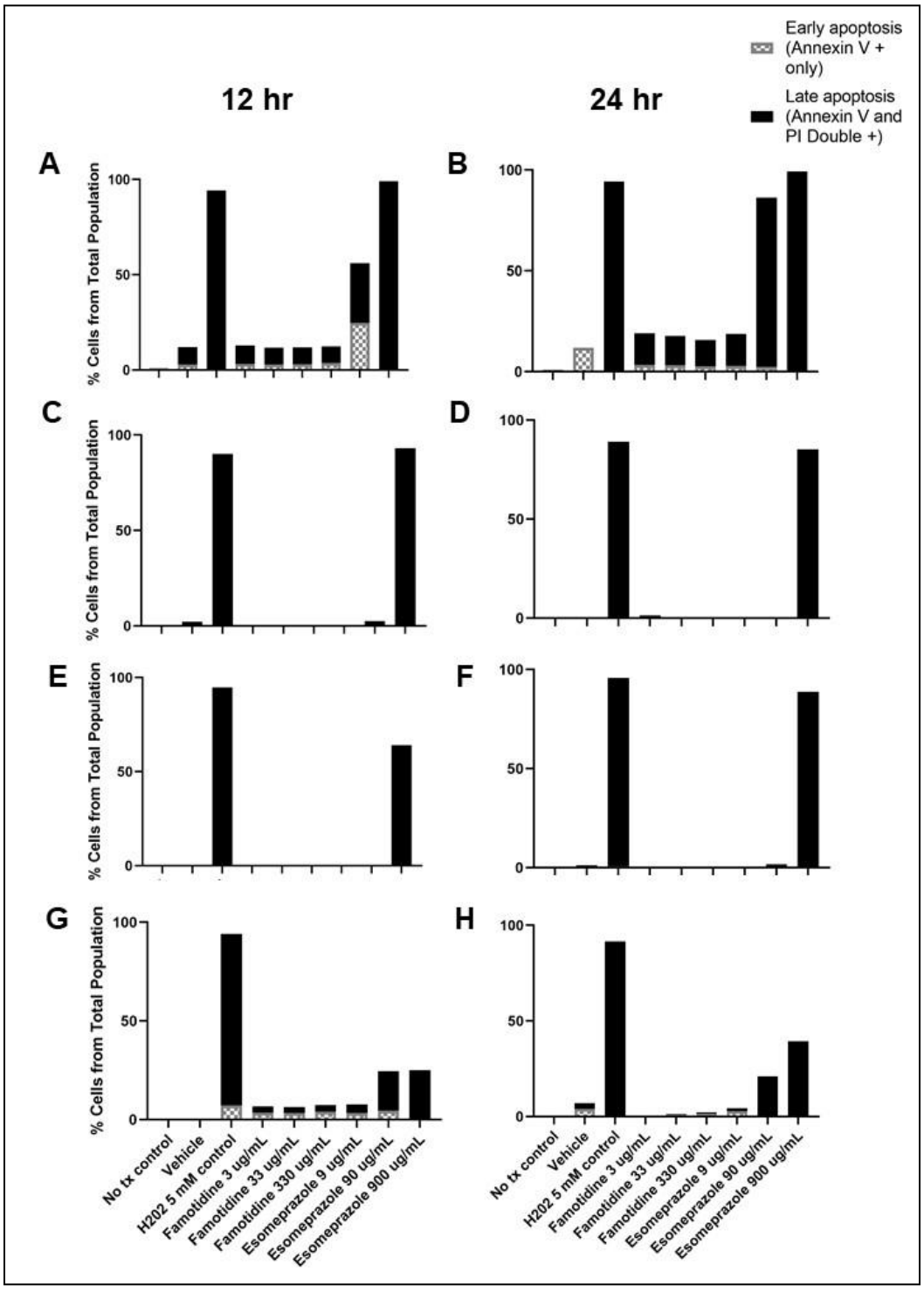

Figure 2.9. **Esomeprazole treatment increases early and late apoptosis compared to vehicle control for both neoplastic MCs and lymphoma cells.** Canine (BR P13 [A,B]; C2 P17 [C,D]) and human (LAD2 P8 [E,F]) MCs exhibited species differences in relative intensities of the treatment effect of acid suppressants at 12 and 24 hours. Note the relative decrease in the percentage of apoptotic cells following esomeprazole-treated 17-71 canine lymphoma cells P9 (G,H). One representative experiment per cell line.

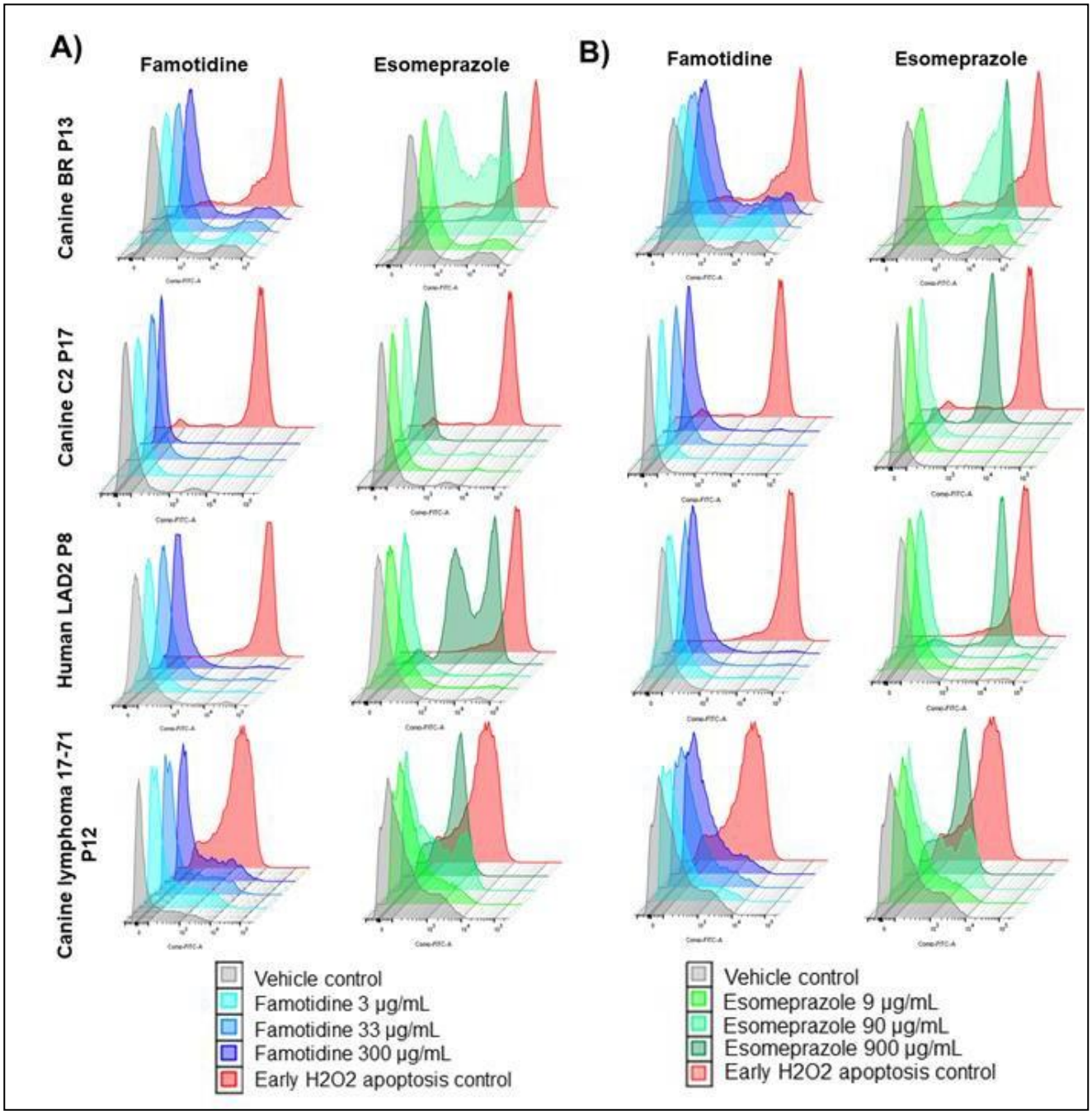

Figure 2.10. Esomeprazole consistently increases early apoptosis in a concentration- and time-dependent fashion in comparison to famotidine and control treatment. Differences in early apoptosis (e.g., Annexin V [FITC-conjugated] positive) fluorescent intensity following 12 (A) or 24 (B) hours of vehicle control, famotidine, or esomeprazole treatment are shown for MCs (canine [BR], human [LAD2]) and canine 17-71 lymphoma cells. The Y axis represents relative amounts of cells per treatment group. Note the consistent lack of treatment effect in all famotidine-treated cells, but clear species and cell specific differences in patterns of apoptosis following esomeprazole treatment. One representative experiment per cell line.

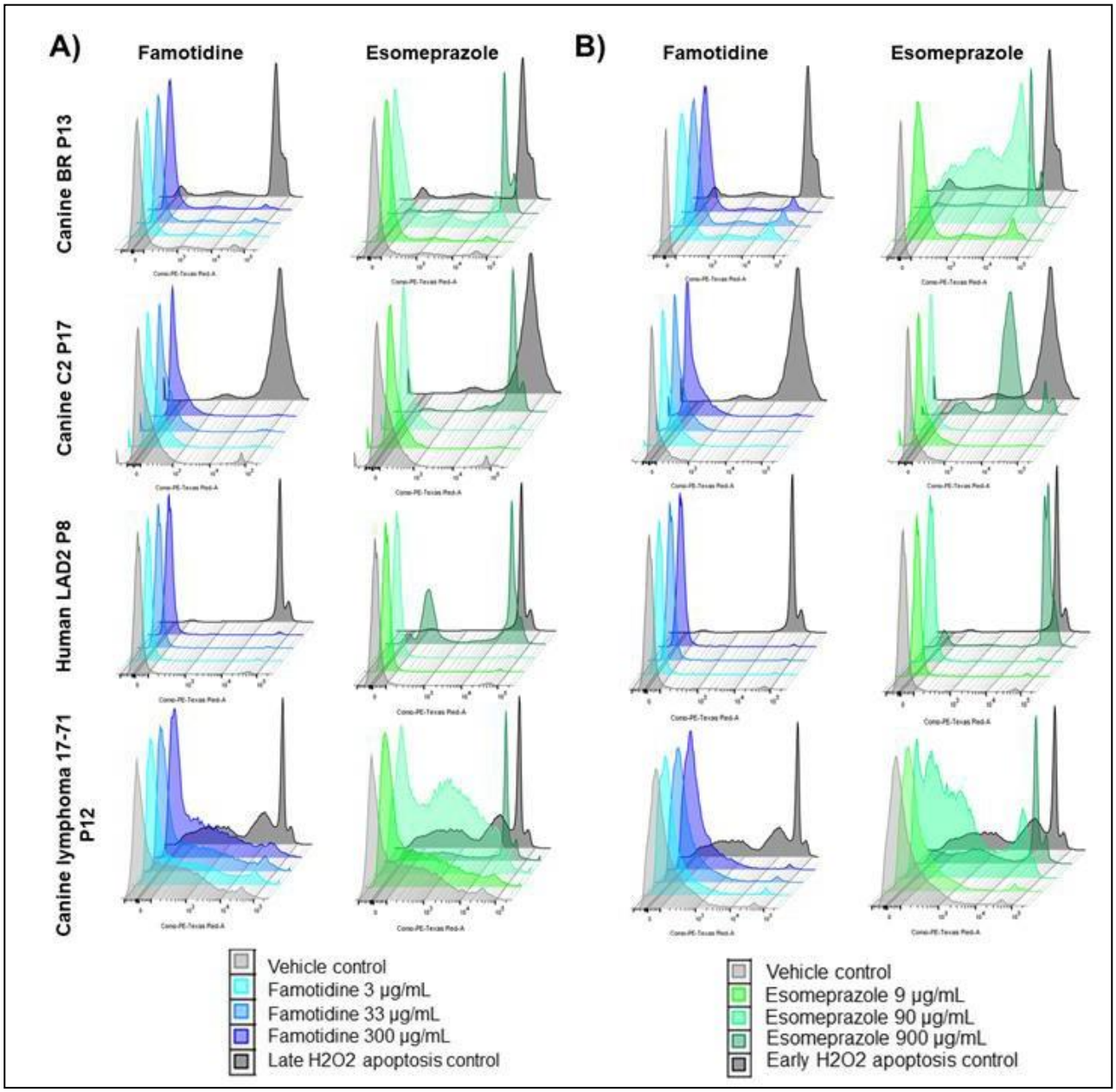

Figure 2.11. Esomeprazole consistently increases late apoptosis in a concentration and time- dependent fashion in comparison to famotidine and control treatment. Differences in late apoptosis (e.g. PI [Texas Red-conjugated] positive) fluorescent intensity following 12 (A) or 24 (B) hours of vehicle control, famotidine, or esomeprazole treatment are shown for MCs (canine [BR,C2], human [LAD2]) and canine 17-71 lymphoma cells. The Y axis represents relative amounts of cells per treatment group. Note the consistent lack of treatment effect in all famotidine-treated cells, but clear species- and cell-specific differences in patterns of apoptosis following esomeprazole treatment. One representative experiment per cell line.

Degranulation

Results of MC activation, as assessed by the measurement of β-hexosaminidase release, found an inconsistent treatment effect of acid suppressants on the LAD2 and RBL-2H3 cell lines. Both significant decreases and increases in degranulation were seen in both cell lines secondary to esomeprazole treatment and stimulation with either A23187, SubP, or IgE stimulation (**Figure 2.12**). Although the canine B cell lymphoma line 17-71 did undergo a yellow color change when read at OD_{490nm} (Gen5 Analysis Software, BioTek® Synergy 2 plate reader), no differences in OD were seen following stimulation with either A23187 or SubP, allowing 17-71 to serve as a negative control for the effects of acid suppressants on MC degranulation.

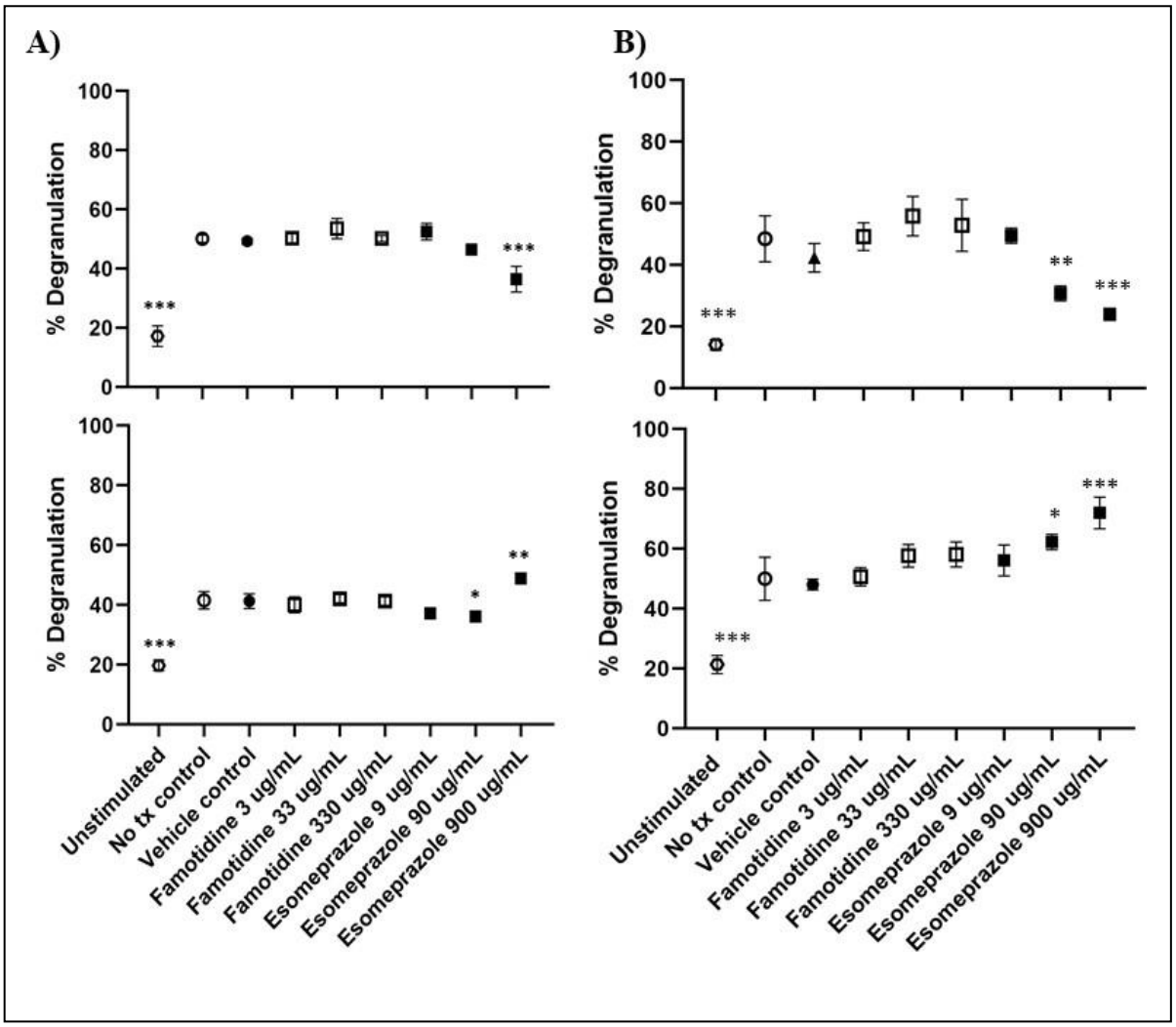

Figure 2.12. Esomeprazole treatment significantly changes MC patterns of degranulation. Human LAD2 (P12) cells stimulated with either 10 µM A23187 for 2 hours (A, top) or 10 µM substance P for 8 hours (A, bottom). Rat basophilic RBL-2H3 (P17) cells stimulated with either 1 µM A23187 for 2 hours (B; top) or 0.1 µg/mL anti-DNP-IgE for 12 hours (B; bottom). All cells were treated with acid suppressants for 12 hours prior to stimulation. Mean +/- SD (% degranulation). Three replicates per treatment group. Passed normality (Shapiro Wilk); One Way ANOVA with Dunnett's post hoc (GraphPad Prism 9.0.2). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. One representative experiment per cell line. Experiments performed in triplicate fashion.

2.4 Discussion

This work is the first to investigate the *in vitro* treatment effects of acid suppressants on canine neoplastic MCs. The aims of our study were to compare effects of a H2RA (i.e., famotidine) with those of a PPI (i.e., esomeprazole) on healthy and neoplastic MC structure, viability, and function.

Similar to previous studies investigating the effects of multiple classes of proton pump (e.g. H+-K+- and V-ATPase) inhibitors on MCs, [4,5] the current study revealed that treatment with a PPI, but not a H_2RA, causes visible concentration- and time-dependent structural changes to MCs as evaluated via both light microscopy and TEM. Significant changes observed include a qualitative decrease in cell size and density as assessed by light microscopy, as well as a significant increase in the amount of cytoplasmic vacuolization present in esomeprazole treated C2 and LAD2 cells. While the mechanism by which esomeprazole induced these changes cannot be elucidated from our study, one reasonable hypothesis is that PPIs bind to V-ATPases in MCs. Inhibition of these pumps by V-ATPase specific inhibitors such as BafA has been found to disrupt MC function.[5] Indeed, the PPI, omeprazole, binds to V-ATPase pumps in the kidney,[18] bone,[18] and purified adrenal chromaffin granules in adrenal glands,[19] confirming a direct interaction between PPIs and extra-GI organ system V-ATPase pumps. Esomeprazole binding to MC V-ATPases could explain some of the structural differences we observed. It is also likely that PPIs induce structural changes to MCs via alteration of other intracellular pathways (e.g., reductions in intracellular calcium flux, *de novo* production of pro-inflammatory cytokines, and MC activation),[4] as direct comparisons of omeprazole and BafA treated murine BMMCs found that PPI treated cells had much more acidic intracytosolic compartments, compared to BafA treated cells.[4] This implies that V-ATPase inhibition is not the only mechanism by which PPIs

exert their treatment effects on MCs. Autophagy is a non-apoptotic, caspase-independent type of cell death that can cause increased intracellular vacuolization. Autophagy is often characterized by an intact cell membrane with increased vacuolization, mitochondrial swelling, and nuclear condensation.[20] This type of cell death is an important catabolic process triggered by the lysosomal degradative pathway, and is important for removal of unneeded or damaged macromolecules.[20] Autophagy has also been identified to occur in MCs (i.e., murine BMMCs, human LAD2).[21,22] Although no significant differences in mitochondrial size were identified between treatment groups in this study, autophagy might account for some of the structural changes seen in one or multiple MC species. Investigation of other mechanisms by which increased intracellular vacuolization occurs in MCs and how this relates to healthy and neoplastic MC function is warranted.

Similar to the differing treatment effects of acid suppressants on MC ultrastructure, esomeprazole, but not famotidine, significantly decreased MC viability, as well increased early and late apoptosis. Interestingly, PPI treatment induced differing amounts of early and late apoptosis in different types of neoplastic MCs, with canine BR cells having a clear and gradual concentration-dependent increase in both early and late apoptosis secondary to esomeprazole. This same type of treatment effect was not observed in human LAD cells, which only developed late apoptosis in response to the highest concentration of PPI. While the canine B cell lymphoma 17-71 line did develop some apoptosis in response to esomeprazole compared to vehicle treatment, it was of a lesser magnitude than any of the MC lines. A consistent finding also specific to the MC cell lines investigated was that while esomeprazole induced significant time and concentration dependent cytotoxicity, famotidine failed to induce cytotoxicity or apoptosis in comparison to vehicle control. Both acid suppressants failed to induce cytotoxicity to canine B

cell lymphoma 17-71 cells, even at the highest concentrations. This collectively suggests that the induction of apoptosis by PPIs are more specific to neoplastic MCs than an agranulocytic, neoplastic line from the same species. We hypothesize that PPIs induce cell death not only via disruption of normal intra- and extracellular pH gradients,[5,23] but also potentially via alterations in normal extracellular signaling pathways that govern kinase activation (e.g., reduced and increased phosphorylation of anti- [e.g., extracellular signal-related kinases; ERKs] and pro-apoptotic [e.g., p38] pathways, respectively).[23] Although *in vivo* pH is tightly regulated for mammalian cell homeostasis and can contribute to apoptosis of healthy cells, neoplastic cells have been documented to be more resistant to changes in the pH of their cell culture media. In one study, progressive acidification of cell culture media was unable to induce neoplastic cell death,[23] despite significant induction of apoptosis of gastric cancer cells secondary to omeprazole treatment, both *in vitro* and in a xenograft mouse model. We therefore think it unlikely that even small, statistically insignificant changes in pH would be a contributing factor to the PPI specific treatment effects seen in our work. To our knowledge, no comparative studies have investigated the mechanisms by which different types of PPIs induce cell death, either programmed or non-programmed, *in vitro* or *in vivo*. It is also unknown how much the degree of MC differentiation might contribute to differing patterns of treatment effects (e.g. well differentiated LAD2s, C2s versus the poorly differentiated BRs). As protease expression differs significantly between well and poorly differentiated MCs, it is reasonable to expect that there are also differences in expression of receptors necessary for activation of apoptosis and necrosis. Future studies investigating species- and tumor-specific differences in the treatment effects of PPIs on neoplastic cell populations are warranted.

Lastly, in contrast to another study that has thus far investigated the effect of omeprazole on MC activation,[4] our findings showed inconsistent treatment effects of esomeprazole on neoplastic *in vitro* MC activation. The effect of H_2RA and PPI treatment on LAD2 MC degranulation, as assessed via percentage of β-hexosaminidase release, was dependent on both the type of stimulator as well as length of treatment for both RBL-2H3 and LAD2 cells. It is possible that the amount of degranulation is also related to MC death (e.g., apoptosis, necrosis), which we found varies depending on length of treatment time and type of MC. Further studies are necessary to investigate the specific mechanisms by which PPIs might be altering pathways necessary for MC activation, including degranulation. An important finding of this study is that, in contrast to the little amount of previously published work that has looked at activation of canine *in vitro* MCs,[13,24] we found both of the canine neoplastic MC lines investigated here to be unreliable models of degranulation. This is largely based on the fact that the optical densities ($OD_{405\ nm}$) of these cells were consistently less than 50-75% than those of the LAD2 and RBL-2H3 cells, indicative of a lack of β-hexosaminidase detection. While the canine C2 line is better differentiated than the BR line, it is one of the least granulated *in vitro* MC lines,[24] logically rendering it an inferior model for the study of degranulation. Although previous studies investigating the activation of BR cells based on measurement of histamine release have found them to degranulate in response to A23187, SubP, and compound 48/80, the percentages of release were always low (e.g. < 20% release).[13] Because we found both the BR and C2 lines were unreliable models for degranulation, conclusions cannot be drawn regarding the effect of acid suppressant therapy on canine neoplastic MCs. To the authors' knowledge, no studies have investigated agranulocytic, *in vitro* cell lines as negative controls for β-hexosaminidase assays. It is unknown if there is cross-reactivity of this assay with other enzymes in mammalian cells,

which would explain the positive color change following incubation of 17-71 cell pellets and supernatants with NAG. Untreated, VC, or acid suppressant treated Tyrode's buffer were negative for the presence of β-hexosaminidase, with very low OD readings (Figure 2.15). Thus, we know that this is not a false positive from an interaction between the acid suppressant compounds and any assay reagents.

Limitations of this study include its *in vitro* design, which might not reflect treatment effects of these drugs in an *in vivo*, canine MCT model. That being said, multiple previous *in vitro* studies documenting similar treatment effects of PPIs have been mirrored by *in vivo* work. Our study also only investigated one method by which to evaluate MC activation via degranulation. Although the use of β-hexosaminidase assays is a well-established method by which to evaluate differences in degranulation patterns,[17] investigation of other methods (i.e., lysosomal associated membrane protein [LAMP]-1 and 2 expression,[25] measurement of prostaglandin [PG] and cytokine release[17]) is warranted. Although the extracellular pH was measured in this study, we did not assess differences between intra- and extracellular pH before and after drug treatment. Because an acidic extracellular pH is important to MC viability and function,[5] as well as advantageous to tumor cell microenvironments, the effect of PPIs on canine neoplastic MC intra- and extracellular pH is worth examining. Lastly, we also looked at only one drug per class of acid suppressant, rather than comparing the treatment effects of multiple types of H_2RAs or PPIs. Because of this, it is important to acknowledge that this study cannot conclude that esomeprazole is superior to all H2RAs, or even other types of PPIs (e.g., omeprazole, pantoprazole, lansoprazole).

2.5 Conclusions

This is the first study to investigate the effects of the acid suppressants famotidine and esomeprazole on neoplastic *in vitro* MC structure, viability, and function. It is also the first study to directly compare differences in treatment outcomes to another canine neoplastic cell line, not of granulocytic origin. Our work demonstrates that treatment of neoplastic *in vitro* MCs from multiple species with esomeprazole, more so than famotidine, alters cell structure, induces significant cytotoxicity, and might alter MC function in response to degranulation stimuli. We found the induction of apoptosis by esomeprazole to be specific to only the granulocytic cell lines studied here. Further studies are necessary to explore the mechanisms by which neoplastic MCs are seemingly more sensitive to the pro-apoptotic effects of PPIs than the canine B cell lymphoma line. Continued *in vitro* and prospective, *in vivo* work comparing the treatment effects of acid suppressants on canine MCTs are warranted.

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CHAPTER III

FUTURE DIRECTIONS (INCLUDING PROSPECTIVE INVESTIGATION OF THE ANTI-INFLAMMATORY AND CYTOTOXIC PROPERTIES OF ACID SUPPRESSANTS ON RESECTABLE CANINE MAST CELL TUMORS)

Objectives: Our primary objective is to evaluate and compare the effects of famotidine and esomeprazole on blood and mast cell tumor (MCT) cytokine production, quantifiable histamine and/or histamine metabolites, and tumor size and viability in dogs with cutaneous MCTs. An additional objective is to evaluate and compare the presence of adverse events (AEs) in dogs with cutaneous MCTs receiving acid suppressants to those receiving placebo alone.

Methods: Eligible study subjects will be randomized to receive either famotidine, esomeprazole, or placebo in combination with diphenhydramine at enrollment. The study will consist of three time points (e.g. initial visit, surgical resection, and post-operative recheck) during which blood and/or tumor tissue will be collected and bio banked. Chemiluminescent multiplex assays and immunohistochemistry will be used to quantify serum and tumor tissue cytokines, respectively. Histamine and histamine metabolites (i.e. N-methylhistamine [NMH]) will be quantified via validated enzyme immunoassays (EIAs or ELISAs) and mass spectrometry. Immunohistochemistry and/or PCR (e.g. terminal deoxynucleotidyl transferase dUTP nick end [TUNEL] labeling, caspase-3 quantification) of tumor tissue will allow for assessment of differences in MCT viability between treatment groups. Tumor size will be monitored throughout the duration of the study, and AEs will be scored according to the Veterinary Cooperative Oncology Group- Common Terminology for Adverse Events (VCOG-CTCAE).

Significance: If PPIs induce significant anti-inflammatory and/or cytotoxic effects, they might be the superior choice in treatment protocols for canine MCT disease. These results will help

optimize efficacious acid suppressant use and potentially minimize morbidity and mortality in canine MCT disease, especially in particularly at risk breeds or populations with metastasis.

3.1 Introduction

Mast cell tumors (MCTs) are the most common skin neoplasm in the dog, comprising almost 1/3 of canine skin malignancies.[1] Common breeds in the United States overrepresented for MCT disease include Boxers, Boston Terriers and the Chinese Shar-Pei.[1,2] One study investigating breed and age associations for common cutaneous neoplasms found Boxers and Boston Terriers to have a 10 and 4.2 times higher odds, respectively, of developing a MCT compared to other breeds.[2] Thus, MCT disease is an important contributor to canine morbidity and mortality, particularly in these purebred populations.

In health, MCs are powerful "first responder" cells containing granules that are loaded with potent mediators such as vasoactive amines (e.g. histamine), heparin, and pro-inflammatory cytokines. These MC mediators are crucial for defense against microbes and recruitment of other immune cells; however, when high numbers of MCs are present together, such as with MCTs, degranulation and release of large amounts of these mediators can have catastrophic consequences. For example, histamine release can trigger angioedema, tissue necrosis, gastrointestinal (GI) ulceration, and tumor-related death. Therapies which focus on mitigating degranulation and its clinical sequela are important to improving quality of life in dogs with MCT disease. For this reason, adjunct therapy for dogs with MCTs includes decreasing gastric hyperacidity and the risk of GI ulceration through the use of gastric acid suppressants, namely histamine-2 receptor antagonists (H_2RAs) and proton pump inhibitors (PPIs). Histamine-2 receptor antagonists block histamine binding to the H2 receptor on the acid-producing gastric parietal cells, while PPIs block the final step in acid production by irreversibly binding to the

H+-K+-ATPase proton pumps responsible for hydrochloric acid secretion. Proton pump inhibitors are currently considered superior treatments for suppression of gastric acid in companion animals,[3] but investigation in canine populations with cancer, specifically MCT disease, has yet to be performed. Despite this, acid suppressants are routinely used prior to surgical resection of the tumor and often included as standard of care for dogs with MCTs. Studies are lacking to support any beneficial effects secondary to administration of these therapies in MCT disease, including impact on circulating histamine and/or reduction in GI-associated adverse events (AEs) (e.g. vomiting, diarrhea, hyporexia or dysrexia).

In addition to overproduction of histamine, MCs secrete interleukins (ILs)-1β, 2, 4, 6, 10, 12, and 33. Interleukin-1β, chemokine CXC ligand 8 (CXCL8), formerly known as IL-8, and tumor necrosis factor (TNF)-α have been recognized as the main inflammatory mediators released by activated MCs.[4-7] The presence of these pro-inflammatory mediators is associated with tumor angiogenesis, metastasis, and local inflammation.[8,9] As previously mentioned, evidence exists that acid suppressants might directly target these inflammatory cytokine pathways, as both H_2RAs and PPIs appear to exert a multitude of effects outside of the stomach. These include modulation of pro- and anti-inflammatory pathways, alteration of leukocyte number, function and viability,[10,11] and reduction in tumor angiogenesis and chemotherapy resistance.[11-15] All of which would be beneficial in neoplastic MC diseases. Though largely derived from *in vitro* work, these findings lead us to believe that H_2RAs and PPIs confer benefits beyond their acid suppressing effects to dogs with cancer, including those with MCTs.

A standardized protocol for the use of acid suppressants in canine MCT disease does not exist. Many veterinarians choose to use both PPIs and H_2RAs together with hopes that they might have a synergistic effect. Although both drugs likely provide benefits, PPIs and H_2RAs

should not be used concurrently when reduction of gastric acid secretion is also desired, as in dogs with MCTs.[16] PPIs accumulate and are activated in the acidic environment of the parietal cell. Concurrent use of H_2RAs decreases the gastric acid suppressing effect of PPIs by decreasing parietal cell acidity and subsequent parietal cell accumulation of PPIs; thus, clinicians must choose the more desirable acid suppressant. Although PPIs are superior to H_2RAs for increasing gastric pH in dogs, H_2RAs are considered to be standard of care for dogs with gross MCT disease with the rationale that they will better mitigate the more systemic effects of histamine release.

In addition to some of the anti-cancer benefits already mentioned above, there is also support that PPIs might directly impact MC viability and function. This is because MC granules depend on a vacuolar ATPase (V-ATPase) pump for maintenance of an acidic pH and MC secretory granule homeostasis.[17,18] A recent study by Pejler et al showed that treatment of *in vitro* mouse bone marrow derived mast cells (BMMCs) with the v-ATPase inhibitor, bafilomycin A, altered histamine storage ability and degranulation profiles and induced structural changes to MC granules.[19] Like bafilomycin, clinically available PPIs such as esomeprazole also disrupt vacuolar ATPase pumps including those in the kidneys and skeleton.[20,21] Another recent study by Kanagaratham et al was the first to demonstrate that treatment of the same BMMCs with omeprazole reduced MC differentiation, degranulation, histamine release and inhibited pro-inflammatory cytokine release.[22] To our knowledge, this was the first work to prove that PPIs have direct effects on MC differentiation and function.

To date, the effect of H_2RAs and PPIs on *in vitro* or *in vivo* canine MCs is largely unknown. *In vitro* work by our group has demonstrated that both acid suppressants have differing effects on structure, viability, and MC activation (e.g. degranulation) in neoplastic

canine MCs.[23,24] We believe that PPI therapy likely has similar effects *in vivo* to what we have demonstrated *in vitro* regarding altered neoplastic MC structure and viability. Thus, a comparative analysis of the *in vivo* effect of acid suppressants on MCTs in dogs is needed to determine which acid suppressant confers the most benefit.

Our central objective is to evaluate and compare the effects of the H_2RA, famotidine, to the PPI, esomeprazole, on blood and MCT cytokine production, quantifiable histamine and histamine metabolites, and tumor size and viability in dogs with cutaneous MCTs. An additional objective is to evaluate and compare the presence of AEs in the same populations of dogs receiving acid suppressants to those receiving placebo. These results will help optimize choice of acid suppressant and potentially minimize morbidity and mortality in canine MCT disease, especially in particularly at risk breeds (e.g. Boxer, Boston Terrier). Results from this study will also serve as a stepping stone for investigation of cytotoxic and anti-inflammatory properties of acid suppressants in patients with metastastic disease, a population in which surgical management is not curative.

3.2 Materials and methods

Subjects and inclusion criteria

Study subjects will be comprised of client-owned dogs ≥1 year of age, weighing ≥2 kg, diagnosed with a surgically resectable, cutaneous or subcutaneous MCTs ≥1 cm in diameter. Minimum tumor size criteria was determined based on the New Response Evaluation Criteria in Solid Tumors (RECIST 1.1) guidelines.[25] Tumor diagnosis will be based on fine needle aspirates (FNAs) evaluated by a board-certified veterinary pathologist. Dogs will be diagnosed either via their referring veterinarian (rDVM) prior to referral, or at initial presentation to the Texas A&M

University (TAMU) College of Veterinary Medicine (CVM) medical oncology, soft tissue surgery, or general practice services.

Treatment groups

All dogs will receive diphenhydramine 1 mg/kg by mouth (PO) twice daily (q12hr) and in addition be randomized via random number generator to receive one of the following three treatment groups: 1) Lactose capsule (placebo) PO q12hr, 2) famotidine (Pepcid AC®) 1 mg/kg PO q12hr) or 3) esomeprazole (Nexium®) 1 mg/kg PO once daily (q24hr) in the morning (AM) + lactose capsule PO q24hr in the evening (PM). Famotidine was selected as it was felt by the investigators to be most common H_2RA administered to dogs with MCTs, and esomeprazole is likely the most potent commercially available PPI for dogs when orally administered at 1.0 mg/kg PO q24h.[26] Additionally, our *in vitro* preliminary studies support the investigation of these specific acid suppressants. All dogs will receive respective treatments orally, administered by owners in their home environment, for the duration of the study.

Study duration/timeline and sample acquisition

Study visits will consist of 3 appointments (i.e. time 1, original baseline visit; time 2, surgical resection of MCT; time 3, post-operative recheck) with a TAMU CVM specialty service, during which blood, urine, +/- FNAs and feces will be collected (**Figure 3.1**). The interval of time between visits will be as standardized as possible between subjects (i.e. time 2 will be 7-10 days following time 1, and time 3 will be 10-14 days following time 2). The total volume of blood drawn will not exceed 15 milliliters (mLs) per visit, which is <10% of subject total blood volume based on a minimum weight of 2 kg. A sufficient volume of plasma and serum will be collected for complete blood count (CBC), biochemistry, isolation of peripheral

blood mononuclear cells (PBMCs), and sample storage for future cytokine, histamine, or histamine metabolite (i.e. N-methylhistamine [NMH]) analysis. Blood will either be allowed to clot and then immediately centrifuged for separation of plasma and serum, or immediately processed for isolation of PBMCs. Following separation, plasma and serum will either be analyzed (CBC, biochemistry) or placed in a Nalgene cryovial and subsequently stored at -80°C for future analysis of other outcome measures.

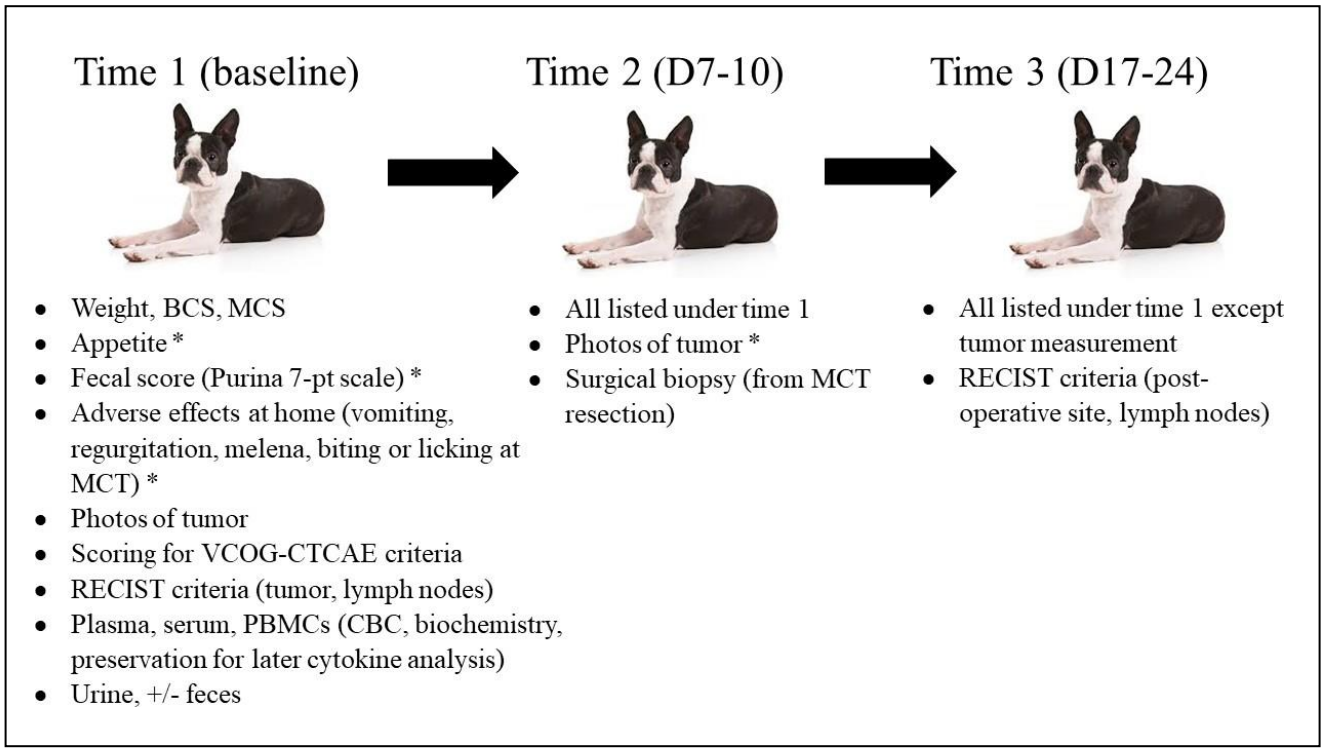

Figure 3.1 Schematic illustrating the projected timeline for study visits and sample collection at each appointment. Time 2 refers to surgical resection of the MCT, and time 3 a post-operative recheck. Parameters denoted with an asterisk (*) will be provided by the owner, either via a questionnaire filled out at time 1, or a log kept at home for the duration of the study and turned in to investigators at subsequent visits. BCS = Body condition scoring; MCS = Muscle condition scoring; VCOG-CTCAE = Veterinary Cooperative Oncology Group- Common Terminology Criteria for Adverse Events; RECIST = Response Evaluation Criteria in Solid Tumors; PBMC = Peripheral blood mononuclear cell.

Isolation of peripheral blood mononuclear cells (PBMCs)

Canine-derived PBMCs will be isolated from EDTA whole blood samples and a commercially available red blood cell (RBC) lysis buffer (eBioscience™ 10x multi-species) used

to separate RBCs from PBMCs. The cell pellet will next be washed a minimum of once in phosphate-buffered saline (PBS) prior to cryopreservation with freezing media (RPMI or DMEM with ≥ 10% heat-inactivated fetal bovine serum [FBS] and 10% DMSO) at -80°C for later analysis (e.g. differences in numbers of PBMCs, markers of apoptosis).

Cytokine analysis

A previously validated, commercially available multiplex electrochemiluminescence assay (Meso Scale Discovery®) will be used to analyze canine interleukins (ILs)- 2 and 6, CXCL8, and tumor necrosis factor-α (TNF-α) in serum or MCT tissue. Other cytokines (i.e. IL-10) can be quantified with individual plates by the same manufacturer. This assay has been validated for measurement of cytokines in canine biological samples, including blood and tissue. All samples will be analyzed in a minimum of 2 replicates per assay. Immunohistochemistry (IHC) is another technique that could be used as an alternate method by which to evaluate for differences in tissue cytokines.

Quantification of histamine and metabolites

Differences in plasma histamine between groups will be quantified either via a validated, enzyme or enzyme- linked immunosorbent assay (EIA; Oxford Biomedical or Cayman Chemical Company Inc, Ann Arbor, MI[22], or ELISA; Immunotech, Marseille, France[27]). Differences in urine histamine metabolites (NMH) will be quantified via mass spectrometry at the Gastrointestinal Laboratory (GI Lab) at TX A&M University.[28,29]

MCT grade and viability

A single board-certified pathologist will grade all biopsy sections from enrolled subjects, as well as assess for differences in MCT death (e.g. apoptosis, necrosis). Immunohistochemical

analysis of tumor tissue for differences in caspase-3 will be used to provide quantitative changes in apoptosis,[30] with amount of necrosis determined based on distinguishing morphologic features visible in MCT tissue sections.

Pre- and post-operative tumor assessment

A quantitative score will be assigned to each tumor or surgical site pre- and post-operatively, respectively, using the response evaluation criteria in solid tumor (RECIST) scoring system.[25,31] This system, which has been validated in both human and veterinary medicine, takes into account local lymph node involvement, and allows for numerical assessment of the surgical site.

Adverse events (AEs)

For the duration of the study, owners will record a daily log of their dog's appetite, fecal consistency (Purina 7-point fecal scoring system[32]), presence of vomiting, regurgitation or melena, and capture photos daily of the mass or post-operative. The presence of adverse gastrointestinal (GI) events will be assessed at each time point (i.e. appointment) and scoring based on a previously validated, standardized scoring system (i.e. VCOG-CTCAE[33]) for AEs in dogs with neoplasia undergoing therapy.

Exclusion criteria

Dogs will be excluded from the study if at any time point the attending veterinarian deems that GI clinical signs (e.g. increased clinical activity index score based on activity, appetite, change in weight, vomiting or diarrhea at a score defined as unacceptable [grade 3 or above for GI AEs in the VCOG- CTCAE, more than 4 continuous episodes of diarrhea classified as a Purina fecal score of 6-7/7], evidence of GI bleeding defined as visible melena,

hematochezia, hematemesis, and development of elevated BUN:Cr ratio with accompanying anemia since study enrollment) worsen from baseline. If subjects develop AEs of grade 3 or above for any category listed in the VCOG- CTCAE or develop an unrelated systemic disease during the study period, this is grounds for reallocation to an unblinded placebo control group (i.e. if AEs felt directly related to study drug rather than underlying disease) or removal from the study. Owners also have the rights to withdraw their dog from the study at any point in time.

Data analysis and statistical design

This is a prospective, double-blinded, randomized, placebo control cohort study. Each continuous response measure will be evaluated for significant differences between treatments and over time using a split plot repeated measures mixed model analysis of variance. Treatment, time, and the treatment-by-time interaction will be treated as fixed effects. Time will be treated as a within subject repeated measure. Animal nested within treatment will be treated as a random effect. Statistical assumptions regarding normality, equality of variances, and outliers will be evaluated for each model using SAS software. Tukey-Kramer p-value adjustments will be applied to post-hoc tests to control for type 1 errors. If statistical assumptions regarding normally distributed residuals are violated, an appropriate transformation will be selected. Categorial response measures will be evaluated with a generalized linear mixed model. If model convergence is not achieved, a generalized estimating equation model (GEE) may be performed. A biostatistician has and will continue to be involved in all aspects of study design, data analysis, and manuscript preparation. As only a minimum amount of comparable research has been performed evaluating our outcome measures of interest in dogs treated with a combination of diphenhydramine + placebo, H_2RA or PPI, we propose collecting an initial sample based on data from a study investigating the effects of acid suppressants on clinicopathologic parameters in

dogs diagnosed with cancer.[34] This will allow us to better assess the total sample size required to detect a 20% change in outcome measures between treatment groups. Assuming a 30% mean change in white blood cell count from baseline, a standard deviation of 2605,[34] an alpha of .05, and power of .8, a sample size of 16 per treatment group (48 total) might be required to detect main effect differences. An interim analysis will be performed after enrolling 10 dogs per group (n=30 dogs total) to determine if additional study subjects are required.

3.3 Other future directions

In addition to this prospective clinical trial which is already underway, another area in which optimization of the use of acid suppressants in companion animal MCT disease is warranted would be dogs and cats with metastatic MCT disease. To the authors' knowledge, no published studies have investigated if dogs or cats with either locally invasive or metastatic MCT disease develop intragastric hyperacidity compared to either healthy pets, those with other systemic inflammatory disorders, or those with other neoplasms. The use of pH capsule continuous monitoring technology (e.g. Bravo™ calibration-free reflux testing system, Medtronic, Minneapolis, MN) allows for comparative investigation of intragastric pH between dogs with MCT disease and healthy controls. This information, combined with endoscopic assessment of the GI tract to evaluate for visible ulceration, will allow veterinarians to assess for if acid suppression is even indicated for these populations in order to control gastric pH. Additionally, while surgical resection is still recommended for metastatic populations with gross disease, if PPIs do reduce circulating pro-inflammatory cytokines and/or are directly cytotoxic to neoplastic MCs, this would help develop a standardized treatment protocol for animals with metastatic disease. These types of studies will better guide efficacious use of acid suppressant therapy in dogs and cats with MCT disease. Because the use of acid suppressants has been shown in some

human populations to reduce chemotherapy resistance,[35] likely due to inhibition of V-ATPase pumps, cytokine milieu, immune response, and subsequent modulation of the tumor microenvironment, future investigation of the use of these drugs in chemotherapy protocols for dogs and cats with MCT disease is warranted. Lastly, investigation of the use of acid suppressants in other systemic diseases with high morbidity and mortality and high amounts of pro-inflammatory cytokines (e.g. immune-mediated hemolytic anemia [IMHA], systemic inflammatory response syndrome [SIRS], and septicemia) is also warranted. Acid suppressants are not currently indicated in any of these disease states unless there is concurrent GI ulceration, bleeding, and/or gastroesophageal reflux disease (GERD).[3] If an acid suppressant were able to significantly reduce pro-inflammatory cytokines, this might improve clinical outcomes for these patient populations and change how we manage these diseases.

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