Autophagy in siRNA-mediated PRKAR1A knockdown canine osteosarcoma cells

by

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A Thesis
Presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Masters of Science
in
Pathobiology

Guelph, Ontario, Canada
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ABSTRACT

Autophagy in siRNA-mediated PRKAR1A knockdown canine osteosarcoma cells

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Canine Osteosarcoma (OSA) is a highly malignant form of bone cancer with a mean survival time of only one year even with aggressive treatment. The poor prognosis of OSA could be due to the ability of OSA cells to resist chemotherapy-induced cell death by entering autophagy. Autophagy, or “self-eating”, is an evolutionarily conserved intracellular catabolic process which plays an important role in adaptation to environmental changes and maintenance of cellular homeostasis. Cancer cells have been shown to enter autophagy and become resistant to chemotherapy; however, the role of autophagy in OSA is currently unknown. Human and canine OSA have numerous similarities including variation in the expression of a gene called PRKAR1A. Patients with low PRKAR1A-expressing OSA have longer post-chemotherapy survival times than high PRKAR1A-expressing OSA in both species. As PRKAR1A interacts with multiple upstream regulators of mammalian target of rapamycin (mTOR), low-PRKAR1A expressing OSA could potentially reduce the autophagic response through mTOR and lead to better chemo-sensitivity. siRNA-mediated PRKAR1A knockdown of canine OSA cells showed a decrease in autophagic response when compared to control cells. However, clonogenic survival assays of treatments showed no statistically significant differences across all treatments. The cytoprotective effects of autophagy could likely be driven by elements more than just the down-regulation of PRKAR1A alone. Future studies are needed to decipher the relationship between autophagy and chemo-resistance of OSA.
ACKNOWLEDGEMENTS

First and foremost, I would like to extend my deepest gratitude and respect to my thesis advisor, Dr. Geoffrey A. Wood. Not only for his continuous support and guidance throughout my master’s degree, but also his willingness in accommodating my bizarre work schedule. His patience and constant reassurance helped me tremendously while I was struggling between medical school applications, extracurricular obligations and research. Under his nurturing supervision, I had the opportunity to strengthen my troubleshooting and analytical thinking skills, which are definitely essential for my medical education. I would also like to extend my immense gratitude to my committee members, Drs. Sarah Wootton and Alicia Viloria-Petit for all the feedback on my thesis dissertation and providing much needed guidance in the development of my experimental design.

I would like to thank all members of the G. Wood’s lab, Drs. Chris Pinelli, Courtney Schott, Emily Brouwer and Ms. Kadi White, for their unending encouragement and assistance throughout my thesis. You helped me see the brighter side of things even in the worst situations and your input was always inspiring. Experiment days were always shorter and a bit easier having you around. Thank you, sincerely.

To my dearest lab technicians, Mary Ellen Clarke, Tami Harvey and Jodi Morrison, thank you very much for your technical support and supervision. Without your expertise, I would not have been able to complete my experiments, let alone graduate on time.

My family have always stood by my side throughout my academic career. I am thankful for my parents, Maureen and Patrick, for giving me the opportunity to resist conformity and explore my own interests, on my own terms. I am also very fortunate to have an older brother, Christopher, who has always been there for me when I needed him.
To my friends, who constantly tolerated my absence for activities and events, thank you for your understanding and kindness. You give me a chance to live a life outside of school. I would especially like to recognize Mei-Hua Hwang, who has the patience to endure my unique sense of humour. I truly appreciate the time we spent together and I am glad for having you as my lab mate and most importantly, my friend.

I would like to thank my girlfriend, Julia King, for all her love and support. You encourage me to pursue my dreams and make me realize that they are achievable. You help me find my long lost confidence and self-esteem by believing in me while there is absolutely no reason to. You are my inspiration for being a good scientist, and a better person.

Last but not least, I would like to thank Ontario Veterinary College Pet Trust for the generous financial support of the project and the Art Rouse cancer biology graduate scholarship for funding my education. I sincerely hope my work will spark further research in the treatment of canine osteosarcoma.
DECLARATION OF WORK PERFORMED

I hereby declare that this thesis is a representation of my original research effort. With the exception of the following item, all work stated in this thesis was performed entirely by myself.

The *PRKARIA*-specific siRNA was developed jointly by Jonathon Liu and Jacqueline Gallienne.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
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<tr>
<td>Akt</td>
<td>Protein kinase B</td>
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<tr>
<td>Ambra1</td>
<td>Activating molecule in Beclin 1-regulated autophagy</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
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<tr>
<td>ATG</td>
<td>Autophagy related gene</td>
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<tr>
<td>Atg</td>
<td>Autophagy related proteins</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic-AMP</td>
</tr>
<tr>
<td>CCA</td>
<td>Cholangiocarcinoma</td>
</tr>
<tr>
<td>CNC</td>
<td>Carney complex</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>DF</td>
<td>Degree of freedom</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DOXO</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Heat-inactivated fetal bovine serum</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506-binding protein of 12 kDa</td>
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</table>
GAP - GTPase activating protein
GDP - Guanosine diphosphate
GEF - Guanine nucleotide exchange factor
GPCRs - G protein-coupled receptors
GRB2 - Growth factor receptor-bound protein 2
GTP - Guanosine triphosphate
HCQ - Hydroxychloroquine
HRP - Horseradish peroxidase
IGF1 - Insulin-like growth factor 1
IMPase - Inositol monophosphataes
KD - Knockdown
LC3 - Microtubule-associated protein light chain 3
L-glut - L-glutamine
LiCl - Lithium chloride
LKB1 - Liver Kinase B1
MAPK - Mitogen-activated protein kinases
MEF - Mouse embryonic fibroblasts
MS - Mean square
MST - Mean survival time
mTOR - Mechanistic target of rapamycin
mTORC1 - mTOR complex 1
mTORC2 - mTOR complex 2
Na3VO4 - Sodium orthovanadate
NCBI - National Center for Biotechnology Information
NCI - Negative control siRNA 1
NSCLCs - Non-small cell lung carcinomas
OSA - Osteosarcoma

FNA - Fine needle aspiration
FS - Full serum
Gua - Guanine
L - Lithium
FS - Full serum
GTPase activating protein
Guanosine diphosphate
Guanine nucleotide exchange factor
G protein-coupled receptors
Growth factor receptor-bound protein 2
Guanosine triphosphate
Hydroxychloroquine
Horseradish peroxidase
Insulin-like growth factor 1
Inositol monophosphataes
L-glutamine
Lithium chloride
Liver Kinase B1
Mitogen-activated protein kinases
Mouse embryonic fibroblasts
Mean square
Mean survival time
Mechanistic target of rapamycin
mTOR complex 1
mTOR complex 2
Sodium orthovanadate
National Center for Biotechnology Information
Negative control siRNA 1
Non-small cell lung carcinomas
Osteosarcoma
PAS ......................................................................................................................... Phagophore assembly site
PBS ......................................................................................................................... Phosphate buffered saline
PDPK1 ...................................................................................................................... Phosphoinositide-dependent kinase 1
PE .............................................................................................................................. Phosphatidylethanolamine
PH .............................................................................................................................. Pleckstrin homology
PI ................................................................................................................................. Lysosomal protease inhibitors
PI3K ............................................................................................................................. Phosphoinositide-3-kinase
PI3P .............................................................................................................................. Phosphatidylinositol 3-phosphate
PIP2 .............................................................................................................................. Phosphatidylinositol 4,5-bisphosphate
PIP3 .............................................................................................................................. Phosphatidylinositol 3,4,5-triphosphate
PKA .............................................................................................................................. Protein kinase A
PMSF ........................................................................................................................... Phenylmethanesulfonyl fluoride
PRKAR1A .................................................................................................................. Regulatory Subunit Type I-α of Protein Kinase A
PS ................................................................................................................................. Phosphatidylserine
PVDF ........................................................................................................................... Polyvinyl difluoride
Raf ................................................................................................................................. Rapidly Accelerated Fibrosarcoma
Rap-1 ............................................................................................................................ Ras-related protein 1
Rheb ............................................................................................................................. Ras homolog enriched in brain
RIPA ............................................................................................................................. Radioimmunoprecipitation assay
RSK ............................................................................................................................... Ribosomal s6 kinase
RTK ............................................................................................................................... Receptor tyrosine kinases
SF ................................................................................................................................. Serum free
siRNA ......................................................................................................................... Small interfering RNA
Sos ................................................................................................................................. Son of Sevenless
SS ................................................................................................................................. Sum of squares
tBl ................................................................................................................................. B lymphoblastoid
TBS ................................................................................................................................. Tris-buffered saline
TBS-T ............................................................................................................................. Tris-buffered saline - Tween 20
TEM ................................................................................................................................. Transmission electron microscopy
TKIs ..........................................................Tyrosine kinase inhibitors
TOP2 ..................................................................Type II topoisomerase
TSC1/2 ..........................................................Tuberous Sclerosis tumour suppressor complex
Ub-like ..........................................................Ubiquitin-like
ULK1/2 ..........................................................UNC-51-like kinases 1 and 2
UVRAG ..................................................UV radiation resistance-associated gene protein
VPS34 ..........................................................Vacuolar protein sorting 34
CHAPTER 1: LITERATURE REVIEW
Canine Osteosarcoma: Overview

Canine OSA is a form of malignant bone tumour in dogs, amounting up to 85% of malignancies originating from bone (Anfinsen et al., 2011) and almost 6% of all canine neoplasia (Priester & Mantel, 1971). It is estimated that over 10000 new cases of OSA arise in dogs in the United States each year (Mueller et al., 2007).

Canine OSA can occur in appendicular (75% of cases), axial skeleton (24%) and extraskeletal (1%) locations (Cavalcanti et al., 2004; Skorupski et al., 2013). Appendicular OSA is more prevalent in forelimbs than in hindlimbs, and forms primarily (75%) in the metaphyseal region of the long bones (McGavin, 2013; Szewczyk et al., 2015). While the proximal humerus and distal radius are the most common primary sites of canine OSA (19% and 14%), it also arises in other appendicular sites such as, proximal and distal femur (6.2% and 8.2%) and proximal and distal tibia (6.6% and 6.2%) (McGavin, 2013; Nunamaker, 1985). Axial OSA, more prevalent in small breed dogs (<30lbs), can manifest in cranium, ribs and spine (Heyman et al., 1992). Extraskeletal OSA is an unusual mesenchymal neoplasm arising in the viscera or soft tissue without involvement of the skeleton (Baba & Cătoi, 2007; Patnaik, 1990).

Metastases

Pulmonary metastasis remains a major life-threatening problem in OSA patients. Due to its aggressive nature, metastasis is very common. 90% will develop detectable nodules within 1 year without appropriate chemotherapy (Wycislo & Fan, 2015). The aggressive metastatic nature of OSA is the result of the early manifestation of micrometastases in which a subpopulation of primary bone tumour cells detach and travel via systemic circulation to secondary locations (Fenger et al., 2014).

Interestingly, the risk of metastasis and mortality is associated with the location of the primary tumour (Szewczyk et al., 2015). In the meta-analysis done by Schmidt et al. in 2013, the authors discovered that OSA at proximal humerus, proximal tibia and distal femur were associated with increased
mortality risk but not metastatic rate. Furthermore, the authors showed a lower metastatic risk for OSA at distal radius. As most studies had multiple regimens of chemotherapy, it was difficult to compare different OSA locations to chemotherapy outcomes. However, it is worthwhile to note that both doxorubicin only and doxorubicin combinations were shown to have significantly better outcome at 5 months post-treatment than other treatments (Schmidt et al., 2013).

Etiology and risk factors

Weight and body size are potential risk factors as larger breeds (>40kg) are more susceptible to OSA than smaller breeds (Bergman et al., 1996; Cooley et al., 2002). It was speculated that extra stress on appendicular skeletons of larger breeds could cause microscopic fractures and chronic inflammation that could induce OSA pathogenesis (Radi & Khan, 2005). However, this remains a speculation as Gellasch et al. showed that increase in body size did not increase micro-damage at the distal radial metaphysis, making weight questionable risk factor of OSA (Gellasch et al., 2002).

Ironically, metallic implants for surgical stabilization of fractures are also associated with increased OSA risk. From studies in World War 1 and 2, imbedded foreign objects such as shrapnel of grenades, mines and shells are known to cause tumour development (Lindeman et al., 1990; Schneider et al., 1997). A retrospective study was done on 13 patients with metal implants from 1956 to 1987 (Sunderman, 1989). Histological analyses from these cases showed increased neoplasms at the site of the implants (Sunderman, 1989). Many of these stainless steel implants were secured by screws of another type of alloy, resulting in extensive damage due to Galvanic and/or fatigue corrosion. Similar findings could be seen in another retrospective study of 35 canine patients where OSA was formed in close proximity to the implantations instead of the common metaphyses of long bones (Verschooten et al., 1980; Sunderman, 1989). The potential carcinogenic properties of the implant material and chronic inflammation from corrosion may lead to tumorigenesis (Sunderman, 1989). Although a direct causation could not be established due to limited primary research and clinical evidence, titanium implants are
currently favoured over traditional stainless steel implants due to superior biocompatibility and fatigue resistance (Dunn et al., 2012).

Another risk factor of canine OSA is gender. The majority studies to date indicate that males are more predisposed to OSA than females. However, this could be associated with gonadal hormone secretion and the age of castration. Studies have shown that dogs that underwent gonadectomy had almost twice the risk of OSA development (Priester & Mantel, 1971; Ru et al., 1998). In Cooley et al., the authors showed that Rottweilers that received gonadectomy before the age 1 had a one in four chance of OSA development and had significantly higher risk to develop OSA than Rottweilers that were sexually intact (Cooley et al., 2002). However, it was also shown that the incidence of OSA in Rottweilers was significantly higher than other types of breed which indicates a hereditary influence (Cooley et al., 2002; Kustritz, 2007). Moreover, experimental studies have shown that exogenous sex hormones can either induce (Bierke & Svedfnstal, 1994; Highman et al., 1981) or suppress OSA development (Fraumeni, 1967; Rooks, W. H., and Dorfman, 1961). In a study by Haraldsson and Nilsson, the authors discovered that mice treated with subcutaneous estrogen injections and radioactive isotope $^{90}$Sr had elevated incidence of OSA (Haraldsson & Nilsson, 1988). In another study done by Highman et al., when compared with the control group, mice fed with dietary estradiol showed no significant statistical differences in OSA incidence (Highman et al., 1981). Due to these conflicting research findings, the exact causality has yet to be established.

Clinical sign and symptoms

Appendicular OSA is generally presented with localized limb swelling and lameness at the primary site (Ehrhart et al., 2013). As previous history of trauma is common among large and giant breeds, the onset of lameness can potentially be misdiagnosed as other orthopedic or tissue damage (Ehrhart et al., 2013). Pain is often associated with periosteal inflammation, micro-fractures or osteolysis of cortical bone at the primary site (Ehrhart et al., 2013). Pain itself could also cause weight loss due to loss of appetite, irritability and aggression (Ehrhart et al., 2013; Liptak et al., 2004).
Symptoms of axial OSA vary depending on the location of primary site. Dogs with OSA originating from ribs could have palpable, painful masses. OSA arising from the mandible or other oral sites could present with swelling, lameness or dysphagia while OSA originating from the cranium could present with facial deformity, exophthalmos and nasal discharge (Ehrhart et al., 2013; Liptak et al., 2004). In more serious cases, neurological symptoms such as hyperesthesia, behavioural change and depression could manifest with OSA at spinal sites (Ehrhart et al., 2013; Liptak et al., 2004).

**Diagnosis**

Initial evaluations include radiographs of the affected area taken both laterally and craniocaudally to differentiate patients from other orthopedic diseases (Ehrhart et al., 2013). The radiographic appearance of OSA can vary from osteolytic to osteoblastic. It usually presents with heterogeneity of both patterns (Liptak et al., 2004). Cortical bone lysis is common among OSA; other pathologic characteristics include abnormal periosteal proliferation, sunburst effect and Codman’s triangle (Liptak et al., 2004). Although patient history, physical examinations and radiographic findings are usually not sufficient for a definitive diagnosis of OSA, veterinarians usually opt for amputation of the affected limb due to the aggressiveness of OSA, lower risk of pathological fracture and immediate improvement of quality of life. A bone biopsy is often performed to confirm the OSA diagnosis. Different approaches such as closed needle or open incisional can be selected based on the patient’s condition and requirements (Desantos et al., 1979; Ehrhart et al., 2013).

In an open incisional biopsy, the surgeon makes an incision at the tumour site previously located on the radiograph and extracts a sample of the tissue. This biopsy option is particularly advantageous for sampling larger piece of tissues which could increase the accuracy of the following histological diagnosis. However, the invasiveness of an open incisional biopsy may increase the risk of post-operative complications such as infection, pathologic fractures and tumour distribution (Desantos et al., 1979; Simon, 1982).
Closed needle biopsy includes fine needle aspiration and closed needle-core biopsy. Fine needle aspiration (FNA), often guided by ultrasound, is a procedure where a thin needle is inserted into a suspected OSA primary site and aspirate a small amount of fluid and cells (Britt et al., 2007). Patients are often given a local anesthetic before the procedure. Although this procedure is minimally invasive, it is rarely used in OSA cases as primary sites are often deep inside skeletal groups where a fine needle cannot reach (Bojrab et al., 2014; Powers, LaRue et al., 1988). In most cases, closed needle-core biopsy would be performed. Since it is a more invasive approach, general anaesthesia is required (Liptak et al., 2004). The veterinarian uses a bone marrow biopsy needle (Jamshidi or Michelle trephine) to extract a core, cylindrical shape tissue from the area (Bojrab et al., 2014). This procedure involves a small area of the tumour site, therefore limiting the risk of neoplasm seeding, fracture, trauma and hemorrhage to surrounding tissues (Bojrab et al., 2014; Ehrhart et al., 2013; Liptak et al., 2004). The accuracy of diagnoses depends on experience and comfort level of the performing veterinarian (Ehrhart et al., 2013; Liptak et al., 2004).

**Standard of Care**

*Amputation*

After the diagnosis, the recommended standard local control for appendicular OSA is amputation. Amputation not only provides pain relief, shortened anesthesia duration, lower risk of complications during surgery and post-operation and lower operative cost (Coomer et al., 2009; Liptak et al., 2004). The drastic approach of amputation also ensures the complete resection of primary tumours and reduces the risk of micro-metastatic diseases by limiting the risk of contamination (Ehrhart et al., 2013). Reports have shown the majority of owners are satisfied with their canines’ quality of life after amputation (Nunamaker, 1985; Withrow & Hirsch, 1979). In some cases, dogs with pre-existing conditions such as obesity, neurological diseases and severe osteoarthritis, as well as owners’ desire to preserve the diseased limb, may opt for limb-sparing procedures (Ehrhart et al., 2013; Straw & Withrow, 1996).
Limb-sparing surgery candidates usually have primary tumours confined to bone, at distal radius and ulna (Ehrhart et al., 2013; Liptak et al., 2004). The candidate should otherwise be in good health and the extent of the primary tumour should also be less than 50% of the entire bone length (Ehrhart et al., 2013; Liptak et al., 2004). Limb-sparing surgery is a less desired approach among most veterinarians due to two major disadvantages. Limb-sparing surgery is often prone to local tumour reoccurrence due to incomplete resection and residual neoplastic cells adjacent to the primary tumour (Ehrhart et al., 2013; Liptak et al., 2004). In Morello et al., it was reported that the risk of reoccurrence from limb-sparing surgery could be up to 28% (Morello et al., 2001). Recipients of limb-sparing surgeries are also at risk of post-operative infections. More than 40% of patients will develop infections and 26% of these cases manifest within 6 months after surgery (LaRue et al., 1989; Morello et al., 2001).

Radiation Therapy

Radiation therapy is administered when OSA patients are not suitable for surgical options and most often used in palliative treatments (Liptak et al., 2004). The primary reasons for palliative radiation therapy are for pain management and to improve quality of life (Mayer & Grier, 2006). Canines with OSA often experience pain from nociceptors through mechanical stimulation and chemical by-products (Mayer & Grier, 2006). OSA could potentially elevate pressure within the primary bone tumour site through activation of osteoclasts which leads to distension of bone, pathological fractures and destruction of sensory nerve fibres (Mayer & Grier, 2006). OSA cells can also generate chemical mediators such as prostaglandins and cytokines which could activate nociceptors and subsequent transfer of pain signals (Mayer & Grier, 2006). Radiation aids in pain management by eliminating some OSA cells, osteoclasts and inflammatory cells (Mayer & Grier, 2006).

Chemotherapy

Chemotherapy is an essential part of a systemic, curative-intent treatment for OSA post amputation due to the aggressive metastatic nature of OSA (Straw et al., 1991); more than 90% of patients have some forms of micrometastatic disease at the time of presentation (Berg, 1996; Straw et al.,
Without chemotherapy, amputation, limb-sparing surgery and radiation have minimal improvements in mean survival time (MST) (Berg, 1996). According to a retrospective study by Thompson and Fugent, the MST of canines treated with limb amputation alone was 168 days with a survival rate of less than 21% at 12 months post-treatment (Thompson & Fugent, 1992). In the same study, canines treated with chemotherapy had an extended MST of 290 days with a survival rate of 36% at 12 months post-treatment (Thompson & Fugent, 1992). Conversely, canines treated exclusively by chemotherapy alone did not contribute significantly to MST (Jaffe et al., 2002; Liptak et al., 2004). In a study by Jaffe et al., out of 31 patients, only 3 patients were successfully cured by chemotherapy alone while the majority of patients had further tumour manifestation (Jaffe et al., 2002). Currently, the most common chemotherapeutic agents for the treatment of OSA are cisplatin, carboplatin and doxorubicin (Szewczyk et al., 2015). These chemotherapies can be used as a single agent or in combination.

**Cisplatin**

Cisplatin is a highly potent and the most widely used chemotherapeutic agent for treatment of human OSA (Berg, 1996; Siddik, 2003). Cisplatin is a platinum-based drug which can react with DNA and induce DNA damage repair mechanism or cellular apoptosis (Siddik, 2003). Cisplatin’s cytotoxicity is activated through intrastrand crosslink adducts of DNA which stimulates multiple signalling pathways such as p53, p73 and MAPK (Siddik, 2003). Cisplatin has shown to be effective in prolonging MST in OSA patients post-amputation. In Straw et al., 39 post-amputated dogs were treated with 2 courses of cisplatin every 21 days while 35 dogs were treated by amputation only as a control group (Straw et al., 1991). Post-operative cisplatin was given to 17 dogs while the remaining 19 dogs received cisplatin pre and post-amputation (Straw et al., 1991). When the MST of the cisplatin-treated groups to MST of the control, it was shown that the cisplatin-treated groups had a significantly prolonged MST compared to control groups (Straw et al., 1991). However, 4% of the treatment groups were euthanized due to metastases (Straw et al., 1991). Another disadvantage with cisplatin is the potential renal toxicity (Berg, 1996). Administration of cisplatin requires constant saline diuresis (Bergman et al., 1996). Although dogs
can generally withstand cisplatin well, even at recommended dosage (70 mg/m$^2$), nephrotoxicity can accumulate over the course of the treatment causing irreversible damage (Berg, 1996; Ehrhart et al., 2013).

**Carboplatin**

Carboplatin is a second generation platinum-based chemotherapeutic agent. Carboplatin is considered to be a safer alternative to cisplatin due to its lower inherent toxicity and less demanding saline diuresis while maintaining a similar clinical effectiveness (Bergman et al., 1996; Calvert et al., 1982). Carboplatin is particularly suitable for canines with pre-existing conditions such as renal diseases and heart complications (Bergman et al., 1996). In Bergman et al., a retrospective study was done on 48 post-amputation OSA patients. These patients were treated with up to 4 cycles of carboplatin, given every 21 days. It was shown that carboplatin was well-tolerated and achieved similar MST to patients treated with cisplatin (Bergman et al., 1996). However, myelosuppression is a well-known toxicity of carboplatin. The adverse effects of carboplatin were studied in Hume et al. where 58 dogs were treated with carboplatin after radiation therapy. 41% of the dogs developed neutropenia and 55% developed thrombocytopenia at 14 days after concurrent carboplatin and radiation therapy (Hume et al., 2009). Although the author failed to establish a statistical significance due to the small sample size and difficulty in differentiating the effects of the radiation therapy to carboplatin, patients treated with carboplatin should be carefully monitored for the development of toxicosis (Hume et al., 2009).

**Doxorubicin**

Doxorubicin belongs to the family of anthracycline drugs and inhibits type II topoisomerase (TOP2) (Nitiss, 2009). By preventing TOP2’s enzyme turnover, the catalytic activity decreases and leads to the accumulation of TOP2-DNA covalent complexes (Nitiss, 2009; Sun et al., 2015). These complexes can induce DNA damage and interfere with normal cellular metabolism (Nitiss, 2009). Doxorubicin has shown to be equally effective as both cisplatin and carboplatin (Szweczyk et al., 2015). The use of doxorubicin is particularly attractive for treatment of canine OSA due to its anti-metastatic effects.
In Berg et al., 35 dogs with appendicular OSA were treated with 5 courses of doxorubicin at a 2-week interval (Berg et al., 1995). Limb amputations were performed between second or third doxorubicin treatment. The MST of the doxorubicin treated group was 366 days with a 1-year survival rate of 50.5%, significantly longer than the amputation only control group (138 days). In another study assessing the effects of BAY 12-9566, a non-peptidic biphenyl matrix metalloproteinases inhibitor, 303 appendicular OSA patients were treated in a doxorubicin administration protocol comparable to Berg et al. The MST for the treatment group was 8 months with a 1-year survival rate of 35%. The most common side effect of doxorubicin is cardiotoxicosis. Although doxorubicin-induced myocardial injury is well documented in human patients, few studies have been done in canines. A study by Mauldin et al investigated the pathological evidence of cardiomyopathy in 175 dogs treated with doxorubicin (Mauldin et al., 1992). Out of these 175 dogs, 32 dogs developed cardiac abnormalities including irregular ECG readings and arrhythmias (Mauldin et al., 1992). 7 dogs were diagnosed with congestive heart failure and died within 90 days post-treatment (Mauldin et al., 1992). However, it is worthwhile to note that the benefits of the treatment outweigh the risk as the MST was extended more significantly than other interventions (Mauldin et al., 1992).

**Autophagy: Overview**

Autophagy, or “self-eating”, is an evolutionarily conserved intracellular catabolic process in which cytosolic proteins, organelles and components are consumed, degraded and recycled to maintain crucial cellular metabolisms and homeostasis (Bolt & Klimecki, 2012; Das et al., 2012; Mathew et al., 2007; White, 2015). Autophagy plays an important role in cellular adaptation to environmental changes and homeostasis regulation by maintaining protein and organelle integrity through degradation of excessive, damaged and/or long-lived cellular components (Fulda et al., 2010; Kimmelman, 2011; Mathew et al., 2007). Autophagy also functions in parallel with the immune system in elimination of apoptotic cells and foreign pathogens (Green et al., 2009; Levine et al., 2011; Mathew et al., 2007). Therefore, impaired or defective autophagy is associated with increased mitochondrial damage, metabolic
Recent studies have shown that autophagy has great influence on physiological and pathological processes (Kenific & Debnath, 2015; Mizushima, 2007). As this cellular process has an intimate relationship with cellular metabolic mediators, autophagy is implicated in various disease pathogeneses, including neuronal degenerative diseases, myopathy, liver disorders and cancer (Levine et al., 2011; Mizushima, 2007; White, 2015). However, it has proven to be very difficult to elucidate the exact mechanism of autophagy in disease pathogenesis due to its broad interactions with other cellular substrates (White & DiPaola, 2009; White, 2015).

Autophagy is sub-categorized into basal and induced (Mizushima, 2007; Musiwaro et al., 2013; Zheng et al., 2012). While basal autophagy occurs at a constant rate in almost all cells and tissues and is primarily responsible for maintaining the quality of the cytosolic components (Mizushima & Komatsu, 2011; Mizushima et al., 2008); induced autophagy is activated during nutrient deficient or stressful conditions to provide alternative energy for survival (Barreto & Geyer, 2014; Russell et al., 2014). Under normal physiological conditions, tissues with high organelle turnover rate such as brain, liver and kidney, are particularly reliant on autophagy to reduce the accumulation of aggregation-prone and unfolded proteins and damaged mitochondria (Mizushima & Komatsu, 2011; White, 2015; Youle & Narendra, 2011; Zhang, 2013). Protein aggregation is often cytotoxic and could potentially lead to neurodegenerative diseases (Takalo et al., 2013). Build-up of damaged mitochondria can disrupt normal cellular homeostasis and release ROS, generating oxidative stress and further destabilizing the cytoplasm (Rabinowitz & White, 2010). Induced autophagy, however, breaks down a wide-range of cytosolic contents to produce energy sources such as amino acids, carbohydrates, lipids and nucleotides; molecular building blocks that can be recycled back into the cytoplasm in response to cellular nutrient starvation and stress (Mehrpour et al., 2012; White, 2015). Once seen as a random cytoplasmic process (Glick et al., 2010; Reggiori et al., 2012), recent evidence has emerged showing that autophagy can selectively remove...

To complicate matters further, different tissues within an organism have specific substrate requirements and may prefer a certain catabolic pathway that produces a particular energy source more than another. For example, liver might favour lipophagy which increases the rate of lipid catabolism in the adipose tissue in order to supply more free fatty acid (FFA) back to the liver (Singh et al., 2009), whereas the brain has been shown to induce ferritinophagy which accelerates ferritin degradation to maintain iron homeostasis (Asano et al., 2011; Chen et al., 2012; Mancias et al., 2014).

While autophagy in normal tissue is context, tissue and substrate dependent, cancer cells have been shown to exhibit the same nature as well. The mechanism by which autophagy modulates tumour growth is currently under extensive investigation. Studies have reported that autophagy can be a promotor or suppressor in cancer pathogenesis (White & DiPaola, 2009; Yang et al., 2011). Most notably the crucial autophagy related gene (ATG)6/BECN1, which is frequently monoallelically lost in human ovarian, breast and prostate cancer (Aita et al., 1999; Choi et al., 2013; Liang et al., 1999). In contrast, loss of ATG7 has been shown to inhibit the growth and metastatic activity of lung tumours in mouse models (Guo et al., 2013), whereas targeted liver-specific autophagy deficiency through deletion of ATG5 and 7 could only produce benign tumours (Takamura et al., 2011). Therefore, the investigational approach towards studying the role of autophagy in tumorigenesis must be context and tissue specific. Although the mechanism of autophagy is extremely complex, exploring these pathways could potentially reveal insights into how to modulate this process towards eventual development of novel techniques and treatments to regulate tumour growth.

*mTOR-dependent autophagy during stress: mechanism of action*

Environment stress, nutrient starvation and lowered circulating growth factors are stimuli that could alert cells to make metabolic alterations that favour survival. These changes in metabolism optimize
the allocation of intracellular energy and nutrient supplies through inhibition of growth and induction of autophagy (Cheong, 2015; Jung et al., 2010). Among other cellular components that regulate and monitor autophagy, the mechanistic target of rapamycin (mTOR) is the fundamental element that balances cell proliferation and autophagy in response to environmental cues (Jung et al., 2010).

mTOR is a serine/threonine protein kinase that integrates growth/nutrient signaling pathways with the autophagy pathway; mTOR is activated by a nutrient/growth factor-sufficient environment to promote anabolic metabolism (Jung et al., 2010; Macintyre & Rathmell, 2011). In contrast, a nutrient/growth factor-deficient environment inactivates mTOR which leads to reduction of cellular energy requirements, growth suppression and induction of autophagy.

mTOR is a central component of 2 distinct cellular complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each with variations of protein components (Gwinn et al., 2008). These complexes regulate autophagy through the integration of signals from upstream growth factor/nutrient signaling pathways and subsequent modulation of downstream targets (Russell et al., 2014). mTORC1 is better characterized than mTORC2 due to its dynamic interplay between multiple signaling networks through monitoring environmental indicators, such as growth factors, energy substrates, and amino acids (Alers et al., 2012; Gwinn et al., 2008).

**Upstream regulators: PI3K-Akt and Ras-ERK pathway**

One of the upstream regulators of mTOR is the growth factor/PI3K/Akt pathway. Growth factors such as insulin-like growth factor 1 (IGF1) and insulin activate the PI3K/Akt pathway through binding of the extracellular domain of receptor tyrosine kinases (RTK). Ligand binding of RTKs induces rapid dimerization leading to autophosphorylation of tyrosine residues of RTKs (Reviewed by Castellano & Downward, 2011). The phosphorylated tyrosine residue is now an available docking site for p85, a subunit of PI3K. The binding of p85 triggers the activation of p110, the catalytic subunit of PI3K (Reviewed by Castellano & Downward, 2011). The activation of PI3K converts the inactive
phosphatidylinositol 4,5-bisphosphate (PIP2) to an active secondary messenger phosphatidylinositol 3,4,5-triphosphate (PIP3) (Reviewed by Castellano & Downward, 2011). The increased level of PIP3 promotes the relocation of pleckstrin homology (PH) domain of phosphoinositide-dependent kinase 1 (PDK1) and the PH domain of Akt to the plasma membrane (Castellano & Downward, 2011; Vanhaesebroeck & Alessi, 2000). By bringing PDK1 and Akt in close proximity, PDK1 phosphorylates and activates Akt (Vanhaesebroeck & Alessi, 2000).

The phosphorylation of Akt activates numerous downstream transcription factors and kinases, including Bcl-2 and MAPK (Kim & Guan, 2015). Most importantly, Akt can negatively regulate Tuberous Sclerosis complex 2 (TSC2) of the Tuberous Sclerosis tumour suppressor complex (TSC1/2) through phosphorylation (Inoki et al., 2002; Jung et al., 2010; Kim & Guan, 2015). TSC1/2 is possibly the most crucial upstream negative regulator of the mTOR-dependent autophagy pathway. TSC1/2 serves as a specific GTPase activating protein (GAP) for the small GTPase of the Ras homolog enriched in brain (Rheb), which is a direct upstream regulator of mTORC1 (Martin et al., 2014). Rheb exists in an active form and constantly stimulates mTORC1’s activity.

Similar to PI3K-Akt pathway, the Ras-ERK pathway is also activated by growth factor binding of RTKs to respond to extracellular and intracellular changes through regulation of cell metabolism and survival by a series of kinase cascades. The activation of the Ras-ERK pathway depends on 2 cytosolic proteins, GRB2 and Sos (Son of Sevenless) (Margolis & Skolnik, 1994). The autophosphorylation of RTK provides a phosphotyrosine binding site for GRB2. GRB2 functions as an adaptor and activator protein for Sos (Castellano & Downward, 2011). Sos, a guanine nucleotide exchange protein (GEF), interacts with the inactive GDP-bound Ras and facilitates with the conformational change to the active GTP-bound form (Lodish et al., 2000b; Margolis & Skolnik, 1994). Activated Ras can then recruit Raf, a serine/threonine (S/T) kinase to the plasma membrane. The relocated Raf is now activated and phosphorylates mitogen-activated protein kinase kinases 1 and 2 (MEK1/2) (Downward, 2003). These kinases continue the phosphorylation cascade and activate the mitogen-activated protein kinases (MAPK)
and ERK (extracellular signal-regulated kinases)1 and 2 (Downward, 2003). Phosphorylated ERK can regulate mTORC1 through TSC1/2 complex in 2 ways. ERK can phosphorylate TSC2 directly or through RSK (Ribosomal s6 kinase) (Zhang & Liu, 2002). Activated RSK interacts with the regulatory site and phosphorylates Ser-1798 at the C terminus of TSC2 (Huang & Manning, 2008).

In a growth factor-deficient environment, RTK dimerization is ceased due to the lack of ligand binding. Both pathways will be unable to initiate downstream signaling without the initial autophosphorylation of RTK. Without the phosphorylation, TSC1/2 complex is no longer inhibited by Akt and ERK (Di Nardo et al., 2014). The GAP domain of TSC2 activates the GTPase of Rheb and facilitates the conversion of the GTP-bound Rheb to an inactive GDP-bound Rheb (Alers et al., 2012). mTORC1’s activity is now diminished without the stimulation of Rheb.

**Upstream regulators: AMPK**

Changes in energy level have also been shown to trigger autophagy (Mihaylova & Shaw, 2011). One of the essential requirements for cellular survival is to balance energy generation and expenditure. The energy sensor of cellular energy level is AMP-activated protein kinase (AMPK) (Mihaylova & Shaw, 2011). By monitoring the ratio of AMP and ATP, the intracellular energy level can be measured, which leads to subsequent modification of metabolism to meet the demand. AMPK is a major negative regulator of mTORC1 (Gwinn et al., 2008). In addition to autophagy, AMPK can regulate cell proliferation and growth through the inhibition of mTORC1 (Mihaylova & Shaw, 2011). AMPK regulates mTORC1 in 2 ways: via direct phosphorylation of mTORC1 and through the TSC1/2/Rheb pathway (Huang & Manning, 2008; Mihaylova & Shaw, 2011).

During energy stress, intracellular AMP increases while ATP declines. The abundance of AMP increases the likelihood of binding to the γ subunit of AMPK (Shackelford & Shaw, 2009). AMP binding leads to a conformational change in AMPK configuration, exposing a binding site at Thr172 of AMPKα (Gwinn et al., 2008; Inoki et al., 2003; Shackelford & Shaw, 2009). This site can now be phosphorylated
by LKB1 (Liver Kinase B1), a tumour suppressor, which activates AMPK (Shackelford & Shaw, 2009). Activated AMPK can directly phosphorylates mTORC1 and TSC2 (Alers et al., 2012).

Raptor is one of the protein components in mTORC1 (Kim et al., 2002). The mTOR-raptor interaction negatively regulates mTORC1 activity (Gwinn et al., 2008; Kim et al., 2002). AMPK directly phosphorylates 2 serine residues of raptor, promotes 14-3-3 binding to raptor and inhibits mTORC1 kinase activity (Gwinn et al., 2008). AMPK can also directly phosphorylate TSC2. However, unlike ERK and Akt in which phosphorylation is inhibitory, phosphorylation of TSC2 by AMPK is excitatory (Mihaylova & Shaw, 2011). The activity of TSC2 is enhanced by AMPK and increases the conversion of active Rheb to the inactive form which leads to the suppression of mTORC1 (Di Nardo et al., 2014; Inoki et al., 2003).

**Downstream targets: ULK1/2, VPS34 and Beclin1**

Much of the early autophagy investigations originated from the yeast *S. cerevisiae* (Russell et al., 2014). Through genetic studies and comparison with other high eukaryotes, several key autophagy-related proteins (Atg) were identified (Alers et al., 2012). Atg1 is among the first autophagy proteins that were identified in yeast (Matsuura et al., 1997). The mammalian orthologs of Atg1 are ULK1 and 2 (UNC-51-like kinases 1 and 2) and are crucial regulators of autophagy. ULK1/2 is a protein complex along with Atg13 and focal adhesion kinase family interacting protein of 200 kD (FIP200) which regulates early steps of autophagosome formation (Hara et al., 2008).

Studies have shown that Atg13 and FIP200 are essential for ULK1/2 activities. Both Atg13 and FIP200 are required for proper autophagosome formation (Alers et al., 2012). Atg13 is a bridge protein that stabilizes and promotes the interaction between ULK1/2 and FIP200 (Roach, 2011). FIP200 is a protein that has multiple cellular functions through its interactions with various proteins such as TSC1, FAK and ASK1 (Hara et al., 2008). FIP200 deficient cells were shown to be incapable of inducing autophagy and impaired ULK phosphorylation (Hara et al., 2008).
Under normal conditions, activated mTORC1 directly phosphorylates Atg13. This phosphorylation of Atg13 prevents the formation of the ULK1/2-Atg13-FIP200 complex (Hara et al., 2008). Inactivation due to nutrient or energy deprivation prevents the phosphorylation of Atg13. Dephosphorylation of Atg13 allows the assembly of the complex and activates ULK1/2 kinase (Alers et al., 2012; Roach, 2011).

The VPS34-p150-Beclin1 complex is directly downstream of ULK1/2 and is the next major regulatory unit of autophagy. The assembly of this complex is essential for autophagosome formation and maturation. VPS34 (vacuolar protein sorting 34) is a catalytic protein of the multiprotein complex which was initially found to be responsible for vacuolar sorting in yeast (Schu et al., 1993). Together with p150, VPS34-p150 forms the class III phosphatidylinositol-3-kinase (PI3K) that regulates autophagy by generating phosphatidylinositol 3-phosphate (PI3P) during early phases of autophagy induction (Jung et al., 2010). Accumulation of PI3P facilitates the formation of autophagosomes and the recruitment of various autophagy proteins required for vesicle elongation (Jackson & Swanson, 2014; Sinha & Levine, 2008). However, the core component of this complex is Beclin 1. Beclin 1 is a Bcl-2-homology (BH)-3 domain only protein which is localized in various cytoplasmic components (Liang et al., 1999; Sinha & Levine, 2008). Beclin 1 is a particularly important element of this complex because of its responsibility for regulating multiple autophagy-related targets, including Atg14, UV Radiation Resistance Associated Gene (UVRAG) and Bax-interacting factor 1 (Bif-1) (Kang et al., 2011). These binding partners have been shown to play a role in autophagosome maturation and mediate fusion of lysosome to the autophagosome (Jackson & Swanson, 2014).

When ULK1/2 kinase is activated, Ambra1 (activating molecule in Beclin 1-regulated autophagy) is phosphorylated (Morris et al., 2015; Yazdankhah et al., 2014). Acitvated Ambra1 binds to Beclin 1 and promotes its translocation to the endoplasmic reticulum (Russell et al., 2014; Yazdankhah et al., 2014). There, Ambra1 facilitates the binding of Beclin 1 to Vps34, therefore allowing the complex to function at the phagophore (Russell et al., 2014; Yazdankhah et al., 2014).
Figure 1: Upstream regulators and downstream targets of mTORC1 under normal conditions
Figure 2: Upstream regulators and downstream targets of mTORC1 under hostile conditions
**Machinery of autophagy**

Core components of autophagy assemble at the PAS (phagophore assembly site) (Wirth et al., 2013). The phagophore, is a structural complex that recruits and stores all the core machinery and Atg proteins necessary for the activation of autophagy (Wirth et al., 2013; Xie & Klionsky, 2007). Although studies of PAS in mammalian systems are currently lacking, much of our knowledge of autophagosome formation can be extrapolated from yeast. Fifteen Atg proteins are recognized as core machinery proteins that drive nutrient-deficient autophagy (Wirth et al., 2013). Most of these Atg proteins are recruited by two key complexes, ULK1/2 complex and the Vsp34-Beclin 1 pathways.

The third key element that drives these downstream events is two unique ubiquitin-like (Ub-like) conjugation systems, the Atg12 and Atg8 systems. In the first system, Atg12 covalently binds to Atg5 and forms a complex with Atg16. Conjugation of Atg12-5-16 is essential for Atg8 lipidation and subsequent autophagosome formation (Walczak & Martens, 2013). The second conjugation system, Atg8, belongs to the LC3 and GABARAP subfamilies. LC3B is the most extensively studied among the Atg8 subfamilies (Nair et al., 2012). Instead of conjugating with another protein, LC3B requires the assistance of the lipid phosphatidylethanolamine (PE) in order to properly function in autophagy (Nair et al., 2012). During nutrient starvation, formation of Atg12-15-6 complex stimulates the conversion of unconjugated LC3B-I to the LC3B-II (PE conjugated) form (Nair et al., 2012; Walczak & Martens, 2013). LC3B-II is then localized to the PAS with the assistance of the Atg12 complex, Atg9 and Vsp34-Beclin 1 complex (Walczak & Martens, 2013). These systems form a cooperative environment and promote expansion of the phagophore membrane.

While the phagophore is undergoing rapid expansion, some cellular components are tagged for degradation. Although the selection process of cellular components for autophagy is still being extensively investigated, p62 has been shown to possess the ability to select damaged proteins that are tagged with ubiquitin (Wurzer et al., 2015). By interacting with LC3B-II on the phagophore, p62 functions as an adaptor protein that links damaged proteins to the phagophore, therefore sealing the fate
of these proteins (Wurzer et al., 2015). p62 has also been shown to assist the curvature of the phagophore expansion, facilitating with the lipidation of proteins (Nath et al., 2014). The two ends eventually meet and fuse, forming a double-membrane autophagosome (Kraft & Martens, 2012). Autophagosomes then fuse with lysosomes and the contents are degraded (Kraft & Martens, 2012).

**Cyclic-AMP dependent protein kinase A: A potential autophagy modulator in canine osteosarcoma**

The induction of autophagy consists of numerous intricate pathways and crosstalk during the process. Due to the wide range of pathways that mTORC1 interacts with, any alteration or influence to the upstream regulators can have a vast impact on subsequent downstream targets and autophagic response. Cyclic-AMP (cAMP) dependent protein kinase A (PKA) is one of these influences on autophagy that recently caught the attention of the scientific community. Currently, the cAMP/PKA signalling pathway is upregulated through ligand binding of G protein-coupled receptors (GPCRs) (Lodish et al., 2000a; Skalhegg & Tasken, 2000). Extracellular signals are transduced through the cell membrane by coupling of G-protein complexes (Skalhegg & Tasken, 2000). These G-protein complexes increase the uptake of GDP and release GTP, which activates downstream target adenylyl cyclase (AC) (Lodish et al., 2000a). AC is a crucial regulator that catalyze the conversion of ATP to cAMP (Lodish et al., 2000a). The increase abundance of cAMP activates PKA, and influences a variety of cellular pathways such as proliferation, gene regulation and metabolism (Skalhegg & Tasken, 2000).

The PKA holoenzyme consists of two main regulatory subunits with different configurations, such as RIα or RIβ and RIIα or RIIβ, with two pairs of catalytic subunits, Ca, Cβ or Cγ (Robinson-White et al., 2006). While all PKA regulatory subunits are involved in cellular processes, RIα is the most abundant regulatory subunit of PKA and is intimately involved in cell cycle progression and genomic stability (Cho et al., 2001; Lutz et al., 2001; Scott, 1991). With the advancement of genotyping techniques, there is growing evidence of the involvement of PRKAR1A (Regulatory Subunit Type I-α of Protein Kinase A), gene coding for RIα, in both human and canine neoplasms.
Carney complex (CNC) is an inherited autosomal dominant disorder characterized by skin pigmentation, myxomas of the heart and breast, and increased risk of endocrine tumours in humans, mostly caused by mutations that lead to the inactivation of PRKAR1A (Bertherat et al., 2009; Sandrini et al., 2002). The majority of CNC patients die of tumour complications due to the aggressive proliferation of myxomas (Robinson-White et al., 2006). In an epidemiological study done by Bertherat et al., 353 patients who carried a germline PRKAR1A mutation or were diagnosed with CNC were studied. At the end of the study timeframe, 32% of the patients developed cardiac myxomas while 41% and 25% developed testicular and thyroid cancer respectively (Bertherat et al., 2009). Preliminary evidence from Adissu et al. demonstrated similar CNC-like symptoms in canines (Adissu et al., 2010).

The tumour-suppressing property of PRKAR1A is also noted in several cancer studies, albeit with varying results and interpretations. In Sandrini et al., thyroid tumours were collected and investigated for R1α level and genetic mutations of PRKAR1A (Sandrini et al., 2002). When compared with benign tumours, thyroid cancers often present with a mutation at the 17q22-24 region. It was also discovered that aggressive thyroid carcinomas have increased PKA activity and decreased PRKAR1A expression (Sandrini et al., 2002). Although the R1α level was lower in thyroid cancers than normal tissues, the study failed to achieve statistical significance and therefore was inconclusive, since the effects could be caused solely by PRKAR1A mutation, independent of protein levels. Another study by Robinsin-white et al. provided an explanation for the strong proliferative nature of cancer in CNC patients. B lymphoblastoid (tBls) cells from patients with CNC that have reduced R1α levels were compared with normal (n-tBls) cells (Robinson-White et al., 2006). It was shown that PRKAR1A inactivated tBls cells had decreased apoptotic response and increased proliferation and survival rate than n-tBls cells under isoproterenol. This study is particularly important as it showed that instead of full inactivation, PRKAR1A inhibition by only 50% was enough to increase cell proliferation.

Although the role of PKRAR1A as a tumour-suppressor is well documented, other studies have also demonstrated that PRKAR1A might be a potential oncogene. Overexpression of PRKAR1A is
implicated in varieties of cancers and could contribute to neoplastic transformation and proliferation. In Loilome et al., human cholangiocarcinoma (CCA) samples were collected and analyzed. Compared to normal adjacent tissues, CCA had an elevated mRNA expression of \textit{PRKARIA} (Loilome et al., 2011). Western blot analyses on four human CCA cell lines also confirmed the strong mRNA expression of \textit{PRKARIA}. In addition, \textit{PRKARIA} knockdown of CCA cells led to reduced proliferation rate and elevated apoptosis response. The most important part of this study was that the author identified that knockdown cell lines had reduced phosphorylation of ERK1/2 and Akt when compared to the untreated CCA cell lines. These results indicated that the knockdown of \textit{PRKARIA} interfered with PI3K/Akt phosphorylation and subsequent activation in CCA cell lines. Moreover, these results also point to the possibility that the effects of \textit{PRKARIA} expression are tissue specific.

Resemblances between the molecular characteristics, tumour pathogenesis, histology and metastatic behaviour of human and canine OSA have been previously established (Mueller et al., 2007; O’Donoghue et al., 2010). Furthermore, hierarchical clustering of OSA expression profiles between human and canine were indistinguishable (Paoloni et al., 2009). Therefore, studies from both species could potentially fill in missing knowledge of the effects of \textit{PRKARIA} in OSA. In Molyneux et al., a data set of previously established OSA microarray data and 20 additional OSA tumour samples were collected. Samples were divided into expression quartiles for low-\textit{PRKARIA} OSA and high-\textit{PRKARIA} OSA and chemotherapy-response data of the two groups were compared. Interestingly, low-\textit{PRKARIA} OSA patients had significantly better responses to chemotherapy than high-\textit{PRKARIA} OSA patients and were 5 times more likely to respond to chemotherapy (Molyneux et al., 2010). Although this study did not test whether there was a correlation between \textit{PRKARIA} expression and overall survival, it indicated that low-\textit{PRKARIA} expressing OSA tumours could potentially be more susceptible to chemotherapy. In canine OSA, a similar study was performed in which 64 OSA samples selected for immunohistochemical staining for \textit{PRKARIA} (Larsen, 2011). Weakly and strongly \textit{PRKARIA} stained samples were separated
and compared in terms of MST. Larsen discovered that canines with low-
PRKAR1A expressing tumours had a significantly prolonged MST than high-
PRKAR1A expressing tumours.

The high metastatic nature of canine OSA makes chemotherapy an important part of the treatment; treatments that could increase the efficacy or lower chemo-resistance of OSA could potentially extend the short MST of OSA patients. It is speculated that increased susceptibility to chemotherapy of low-PRKAR1A expressing OSA is due to the inability of these cells to elicit a chemo-protective autophagic response. This hypothesis is highly plausible as there is extensive crosstalk between PKA and MAPK pathways, and the molecules involved are intimately associated to mTORC1 regulation.

The interactions between PKA and MAPK pathways are highly complicated and context specific. Studies have shown that PKA and MAPK pathways were co-activated in the maturation stage of neuritogenesis (Vogt Weisenhorn et al., 2001). Additional studies have also shown the accumulation of cAMP directly inhibits growth factor induced activation of the MEK/ERK pathway in in vitro settings (Burgering et al., 1993; Cook & McCormick, 1993; Graves et al., 1993). RAS was also found to be involved in cAMP-induced activation in an in vitro study using PKA inhibitors (Ambrosini et al., 2000; Pham et al., 2000). Indirectly, PKA was shown to be able to inhibit MEK1/2 and ERK1/2 through the activation of Rap-1, an inhibitor for both targets (Lerosey et al., 1991; Schmitt & Stork, 2001).

Furthermore, PKA mediated phosphorylation of RAF-1 through cAMP accumulation can also activate Rheb (Karbowniczek et al., 1997). The inhibitory effects of PKA, acting through key regulators of mTOR could decrease mTOR activation and induce autophagy activation. In this scenario, low-PRKAR1A expressing OSA could have better post-chemotherapy response because of reduced autophagy from less inhibitory interactions from PKA. In Mavrakis et al., prkar1a +/+ mouse embryonic fibroblasts (MEF) cells and prkar1a −/− MEF cells were compared in terms of number of autophagosomes and mTOR activity. Not only did prkar1a −/− MEF cells have less total autophagosomes, but western blot analysis showed that these cells also had the highest p-mTOR/mTOR ratio (Mavrakis et al., 2006). These results indicated that PKA could be linked to regulation of autophagy through mTOR signalling.
**Autophagy and cancer therapy**

More investigations are still needed to decipher the effects of autophagy on OSA during chemotherapy. Many studies have shown that autophagy is a primary protective mechanism for cancer cells and could contribute to chemo-resistance (Maycotte & Thorburn, 2011). However, conflicting studies have made clinical trials extremely slow to proceed as others studies have also shown the complete opposite of the effects of autophagy. In a recent study by Suzuki et al. testing the efficacy of the combination chemotherapy genistein and 5-fluorouracil to treat pancreatic cancer, it was shown that both apoptotic and autophagy-related cell death were enhanced with an elevated beclin-1 levels (Suzuki et al., 2014). In addition, Wei et al. explored the importance of autophagy in non-small cell lung carcinomas (NSCLCs) treated with tyrosine kinase inhibitors (TKIs) like gefitinib and erlotinib showed that autophagy suppression both in vivo and in vitro reduced the efficacy of TKIs treatments (Wei et al., 2013). Therefore, clinical trials on cancers with combination treatments similar to Suzuki et al. and Wei et al. which utilize autophagy inhibitors such as hydroxychloroquine (HCQ), in conjunction with chemotherapy, could be ineffective and potentially detrimental.

Manipulating autophagy has immense clinical potential in cancer therapy; however, the current crucial priority is to identify which cancers will benefit from autophagy inhibition or activation. By characterizing cancers in terms of how autophagy affects chemotherapy efficacy, targeted therapeutic approaches could be developed, and the best combination of chemotherapy and autophagy modulators determined.
RATIONALE, HYPOTHESIS, AND OBJECTIVES

Osteosarcoma (OSA) is a highly malignant type of bone tumour that affects both humans and canines. These tumours have similar genomic makeup, pathogenesis and histological presentations in both species, and there is also evidence for shared clinical potential in the expression of PRKARIA. Low PRKARIA-expressing OSA has been associated with better clinical response and outcome when compared to high PRKARIA-expressing OSA in both species. Modulation of PRKARIA could be a possible therapeutic target due to its intimate interactions with the mTOR pathway, and lower expression of PRKARIA may decrease autophagic response in OSA and lead to increase chemo-sensitivity. To investigate this concept, this thesis explores the autophagic response of canine OSA cell lines under chemotherapy by downregulating PRKARIA expression using siRNA.

Two primary canine OSA cell lines will be used for all experiments. JL-31 is a pulmonary metastatic OSA cell line collected from the thoracic fluid and JL-75 was derived from distal tibia OSA. PRKARIA specific siRNA was previously developed and will be used to knockdown PRKARIA expression levels in these cell lines by transfection. 3 different serum conditions will be used to emulate different cellular environments; normal culture media and serum free media will be used as nutrient abundant and deficient environments respectively. Doxorubicin treated media will be used as the chemotherapy-stress condition. Autophagy modulators such as rapamycin and hydroxychloroquine will be utilized as experimental controls.

Various analytical techniques will be used to determine autophagic, apoptotic responses and proliferation ability post-chemotherapy treatment. Western blot analyses targeting LC3-I and II and p62 will be used to determine autophagic response at the protein expression level. LC3-specific GFP will be used to visualize the autophagic substrates through fluorescence microscopy. Clonogenic assays will be performed after treatments to evaluate the colony forming ability of cells.
We hypothesize that *PRKARIA*-downregulated cell lines will have increased chemotherapy-induced apoptosis and reduced autophagic response.

**Objective 1:** R1α KD suppresses autophagy of canine OSA cell lines under chemotherapy-stress, leading to increased cell death

**Objective 2:** Suppression of autophagy through R1α KD is mTOR dependent
CHAPTER 2: AUTOPHAGY IN siRNA-MEDIATED PRKAR1A KNOCKDOWN CANINE OSTEOSARCOMA CELLS UPON EXPOSURE TO CHEMOTHERAPEUTIC AGENTS
Introduction

Osteosarcoma (OSA) is a highly malignant form of bone tumour which accounts for 85% of skeletal tumours in canines (Anfinsen et al., 2011). Canine OSA most commonly affects appendicular skeleton such as proximal humerus and distal radius (Cavalcanti et al., 2004). Although less prevalent, canine OSA can also manifest in axial and extraskeletal locations (Nunamaker, 1985). While an estimated 10,000 canines are diagnosed with OSA each year in the United States, a significant portion of these cases are from large and giant breed dogs, including Great Dane, St. Bernard and Rottweiler. It is speculated that the increased stress on appendicular skeletons in heavier breeds could cause microscopic fractures and chronic inflammation that lead to OSA formation (Radi & Khan, 2005). The standard of care for canine OSA is often amputation of the affected limb, or limb-sparing surgery, prioritizing a complete resection of the primary tumour (Ehrhart et al., 2013). However, canines that undergo limb amputation alone have a 1-year survival rate of less than 21% (Thompson & Fugent, 1992). For a curative-intent treatment plan, chemotherapy is often administered after amputation (Rodney C. Straw et al., 1991). Yet, even in canines treated with amputation followed by adjuvant chemotherapy, the 1-year survival is still a measly 32%. The poor prognosis of canine OSA is due to its aggressive metastatic potential (Wycislo & Fan, 2015). Chemo-resistant micrometastases often manifest early during OSA pathogenesis; despite aggressive treatment interventions, more than 90% of patients develop metastases (Ehrhart et al., 2013). Moreover, compared to its human counterpart, current treatments of canine OSA are relatively ineffective in terms of survival rate (Withrow et al., 1991). New treatment approaches are needed to improve the devastating prognosis of this disease.

One factor that could contribute to the chemo-resistance of canine OSA is its innate ability to undergo autophagy. Autophagy is an highly conserved intracellular catabolic process in which upon activation, cytosolic components are degraded and recycled to produce energy to maintain crucial cellular functions (Mathew et al., 2007; White, 2015). This catabolic process is particularly important in maintaining protein and organelle integrity under stressful conditions, such as nutrient deficiency,
hypoxia and presence of chemotherapy agents (Fulda et al., 2010; Karantza-Wadsworth et al., 2007; Kimmelman, 2011). By converting damaged mitochondria, cytotoxic protein aggregates and other cytosolic components to energy, autophagy promotes cell survival by supplying cells with alternative energy sources while restricting necrosis and inflammation (Rabinowitz & White, 2010; Takalo et al., 2013; Z. J. Yang et al., 2011). Like normal cells, canine OSA cells could potentially exhibit similar autophagic responses rendering them resistant to the cytotoxicity of chemotherapy agents.

Autophagy is monitored and regulated by the mechanistic target of rapamycin (mTOR), an essential cellular component that balances cell proliferation and survival according to environmental cues (Jung et al., 2010). Located directly downstream of various important regulators such as PI3K-Akt, Ras-ERK and AMPK pathways, mTOR integrates signals from these pathways and modulates downstream targets (Russell et al., 2014). These downstream targets, such as Atg13, ULK1/2 and Beclin-1, are often crucial for the induction of autophagy. Therefore, interference with any upstream components of mTOR could have drastic effects on the autophagic response. A potential molecule that could have such impact is the cyclic-AMP (cAMP) dependent protein kinase A (PKA).

Activation of PKA through increased intracellular cAMP influences numerous cellular pathways that are vital to gene regulation, proliferation and metabolism (Skalhegg & Tasken, 2000). PKA consists of 2 main regulatory subunits (RIα or RIβ and RIIα or RIIβ) and 2 pairs of catalytic subunits (Cα, Cβ or Cγ) (Robinson-White et al., 2006). The regulatory subunit RIα, encoded by PRKAR1A (Regulatory Subunit Type I-α of Protein Kinase A), is the most abundant form of PKA and mediates and cell cycle progression (Lutz et al., 2001; Scott, 1991).

Both human and canine PRKAR1A share striking similarities in genetic sequence, as well as disease manifestations when mutated. Carney complex (CNC) is an inherited autosomal dominant disorder that causes heart, breast and endocrine tumours due to the inactivation of PRKAR1A in humans.
(Sandrini et al., 2002). Similar to humans, inactivation of \textit{PRKARIA} in canines could manifest in CNC-like symptoms (Adissu et al., 2010).

The overexpression of \textit{PRKARIA} has been identified to contribute to neoplastic transformation and proliferation. Therefore, it is a reasonable prediction that a therapeutic approach that lowers \textit{PRKARIA} could potentially be clinically beneficial. In a human OSA study, it was found that patients with low-\textit{PRKARIA} expressing OSA responded better to chemotherapy than patients with high-\textit{PRKARIA} expressing OSA (Molyneux et al., 2010). Similar results were also discovered in a canine study where were protein levels were analyzed following immunohistochemistry (Larsen, 2011). It was discovered that low-\textit{PRKARIA} expressing tumours had a significantly prolonged mean survival time (MST) than high-\textit{PRKARIA} expressing tumours. As \textit{PRKARIA} interacts closely with the mTOR pathway, low-\textit{PRKARIA} expressing OSA might translate into a reduced autophagic response and therefore are more susceptible to chemotherapy.

This study investigated whether siRNA-mediated knockdown (KD) of \textit{PRKARIA} inhibited the autophagic response in canine OSA cells. Given that autophagy is implicated in cytoprotective effects, we further explored the possibility that the down-regulation of \textit{PRKARIA} could lower chemo-resistance. We discovered that doxorubicin-treated \textit{PRKARIA} KD cells had increased LC3-I expression levels and reduced LC3-II levels, suggesting an inhibition of autophagy. However, this finding did not translate to an increase of chemo-susceptibility in clonogenic survival assays where KD cells showed no statistical significant difference when compared to control cells across all treatments. These results suggested that inhibition of autophagy by \textit{PRKARIA} KD was not enough to elicit any clinical advantages. Further investigation is required to explore the effects of \textit{PRKARIA} expression in canine OSA.
Materials and methods

Explants of primary canine OSA cell lines

Two canine OSA cell lines were previously derived from OSA cases received at the Ontario Veterinary College. JL-75 cell line originated from a distal tibia tumour, whereas JL-31 was derived from a metastatic lung tumour. Both samples were extracted and processed in the post-mortem room at the Veterinary Teaching Hospital, University of Guelph, Ontario. Although the two cell lines were processed at different times, the tissue preparation method was identical. Extracted tissues were sanitized with 70% ethanol for 30 seconds and then washed with phosphate buffered saline (PBS). Tissues were then plated in a 10cm tissue culture dish contained 10mL of Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, supplemented with 500μg/mL gentamycin, 10μg/mL fungizone and 10% heat-inactivated fetal bovine serum (FBS) (Fisher Scientific, Whitby, ON). Within 10 minutes of extraction, the dishes were placed into a laminar flow hood for further processing. Tissues were minced into 2-3mm fragments and placed into 25cm² cell culture flasks with DMEM high glucose, 10% heat-inactivated FBS, 50μg/mL gentamycin and 1μg/mL fungizone. The flasks were then cultured at 37°C with 5% CO₂.

Maintenance of cell lines

Both cell lines were maintained in DMEM high glucose supplemented with 10% FBS and 1% L-glutamine (Fisher Scientific, Whitby, ON). Media was aspirated and replenished every 3-4 days until cells reached 85 – 90% confluency. Cell were harvested and passaged by adding 1mL of 0.25% Trypsin-EDTA (Life Technologies, Burlington, ON) for 5 – 10 minutes to allow full detachment and achieve a single cell suspension. Deactivation of Trypsin was accomplished by resuspending in culture media, 5 - 10 times the amount of Trypsin. Cells were then transferred to 4 culture dishes and resuspended in culture media. Again, once grown to 85 – 90% confluency, cells were harvested and passaged as previously described. All experiments were done in cells with passage numbers between 2 to 7. At passage 1 to 3, cells were harvested and resuspended in culture media. Cells were centrifuged at 1500rpm for 5 minutes. Cells were then resuspended at 2.5 - 5 x 10⁵ cells/mL in culture media and supplemented with additional FBS (20%)
and 10% dimethyl sulfoxide (DMSO) (Fisher Scientific, Whitby, ON). Cells were aliquoted into cryogenic vials and transferred into a Mr. Frosty™ Freezing Container containing 95% isopropanol (Fisher Scientific, Whitby, ON). The container was placed in a -80°C freezer for 24 hours and then transferred a liquid nitrogen tank for storage.

**Cell viability assay**

Cell viability was assessed using the PrestoBlue® cell viability reagent (Life Technologies, Burlington, ON), a resazurin-based solution that is used as an indicator of cellular metabolism. The assay detects oxidation-reduction reactions, in cells that absorb the naturally blue resazurin and reduce the reagent into resorufin, which is pink and highly fluorescent. The fluorescence intensity is directly proportional to the number of metabolically active cells. By measuring the fluorescence intensity, the effects of treatments on cellular viability could be quantitatively measured.

**Determining Doxorubicin IC$_{50}$**

JL-31 and J1-75 cell lines were plated in triplicate at a density of 4000 or 5000 cells per well in a 96-well plate and let to attach for 24 hours. Cells were treated in quadruplicate with 100μL of serial dilutions of doxorubicin from 0.05μM to 100μM, diluted in culture media. The plates were then incubated at 37°C with 5% CO$_2$ for 72 hours. After the incubation, 11μL of the PrestoBlue® cell viability reagent was added into each well. The fluorescence intensity measurements were captured by the BioTek Synergy HT plate reader with Gen5 version 1.11 software (BioTek, Winooski, VT). The excitation and emission fluorescence were collected at 535nm and 645nm. The data was normalized by subtracting the mean fluorescence measurements of the treatment wells from fluorescence measurements of wells with media or treatment only (no cells).

**siRNA transfection**

The siRNA was previously designed by a former graduate student in the lab (Gallienne, 2014). First, the target *Canis lupus familiaris PRKAR1A* gene (Accession number: XM_537577.4) was located
using the National Center for Biotechnology Information (NCBI) RefSeq Genbank. The siRNA was then designed using the Predesigned DsiRNA Selection Program (IDT Technologies, Coralville, IA). The resulting product was a vial of 27mer duplex RNAs targeting canine PRKAR1A gene. The siRNA was then aliquoted to the stock concentration of 2μM using Opti-MEM® Reduced-Serum Medium (Fisher Scientific, Whitby, ON). The siRNA was further diluted with Opti-MEM® to a final concentration of 40nM before transfection. The cells were then transfected with the siRNA using Lipofectamine 3000, according to the manufacturer’s instructions (Life Technologies, Burlington, ON). Within the same plate, 1 well of each cell line was transfected with a scrambled universal negative control siRNA duplex (NC1 negative control siRNA; Integrated DNA Technologies, Coralville, IA), a scrambled sequence of the designed siRNA. The transfected cells were incubated for 48 hours before treatments.

Treatment protocols

Both cell lines were seeded at a density of 8 x 10^4 cells per mL in a 6-well or 12-well plate. Cells were grown in standard culture media and incubated at 37°C with 5% CO_2 for 24 hours. Cells were then transfected either with PRKAR1A siRNA or NC1, according to transfection protocol described previously. After 48 hours, media was aspirated and cells were washed with 1X PBS. PBS was then removed and treatments, diluted in standard culture media, were added into each well. The plates were incubated for 24 hours. Cells were then harvested and analyzed by western blotting, clonogenic assay, fluorescence microscopy and flow cytometry, as described below.

Western Blot Analysis

Protein collection and quantification

Cells were seeded, grown and treated according to treatment protocol. After treatments, dishes were transferred to the laminar flow hood. Media was then removed by aspiration and wells were rinsed with cold PBS. 1mL of working radioimmunoprecipitation assay (RIPA) buffer was made by combining 898μL of RIPA buffer, 100μL of 10X protease inhibitor cocktail, 1μL of 1mM phenylmethanesulfonfyl
fluorid(PMSF) and 1μL of 1mM sodium orthovanadate (Na₃VO₄)(Sigma-Aldrich, Oakville, ON). Dishes were then placed on ice and working RIPA buffer was added into each well. The volume of working RIPA buffer added was approximately at a ratio of 50μL to 1mL working volume of the well. Cells were scraped off the dishes using cell scrappers and transferred into 1.5mL Eppendorf tubes (Fisher Scientific, Whitby, ON). Samples were placed on ice for 15 minutes and vortexed every 2 minutes to ensure uniform lysis. Samples were then centrifuged at 14000rpm at 4°C for 20 minutes. Supernatants were transferred into new Eppendorf tubes and stored at -80°C. Sample total protein concentrations were quantified using a Bradford assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a reference protein. Serial dilutions of BSA were prepared and Bio-Rad Protein Assay Reagent was added into the dilutions (Bio-Rad Laboratories, Mississauga, ON). A standard curve was generated by plotting the corresponding absorbance shift of each dilution. The standard curve was then used to determine the total protein concentration of each sample according to the absorbance level.

**Western Blotting**

2X reducing buffer was first prepared by combining 5mL 2x Laemmli Sample Buffer and 270mg Dithiotheitol (DTT) (Bio-Rad, Mississauga, ON). Depending on the number of wells, 10μg (15 wells) or 30μg (10 wells) of total protein was mixed with 2X reducing buffer and water, then heated to 95°C for 15 minutes. Treated protein samples and a PiNK Plus Prestained Protein Ladder (FroggaBio, North York, ON) were loaded into wells of 12% TGX Stain-Free™ FastCast™ Acrylamide gels (Bio-Rad, Mississauga, ON). Submerged inside the gel chamber with 1X Tris-Glycine SDS running buffer (Bio-Rad, Mississauga, ON), the proteins were separated by molecular weight at 200V for approximately 45 minutes or until the dye front ran off the gel. The gel was then placed on a methanol-activated polyvinyl difluoride (PVDF) membrane and sandwiched in between 2 transfer stacks (Bio-Rad, Mississauga, ON). The stack was then transferred to the Trans-Blot® Turbo™ Transfer System and moistened with 1X transfer buffer (Bio-Rad, Mississauga, ON). The proteins were transferred to the membrane at 25V for 3 minutes. After the transfer, the membrane was washed in 1X tris-buffered saline (TBS) (Fisher Scientific,
Whitby, ON) containing 0.5% Tween 20 (TBS-T), twice for 10 minutes on a Belly Dancer™ undulating orbital shaker (Sigma-Aldrich, Oakville, ON). The membrane was then blocked in TBS-T with 5% (w/v) skim milk for 1 hour at room temperature. After blocking, the 5% skim milk was removed and the primary antibody, diluted in 5% skim milk TBS-T, was added. The membrane was then incubated at 4°C on a rocker overnight. On the following day, the membrane was washed in TBS-T twice for 10 minutes. The secondary antibody conjugated with horseradish peroxidase (HRP) was diluted in 5% skim milk TBS-T, was applied to the membrane and incubated for 1 hour. After 2 TBS-T washes for 10 minutes, the membrane was washed in TBS for an additional 10 minutes. Next, Clarity Western ECL substrate (Bio-Rad, Mississauga, ON) was added on to the membrane and incubated for 5 minutes. Chemiluminescence signals were detected by the ChemiDoc™ XRS+ Imager with Image Lab software (Bio-Rad, Mississauga, ON). Additional quantification of bands was performed by densitometry on the Image J software.

**Determination of autophagic flux**

**Lysosomal protease inhibitors**

Autophagic flux was determined by the conversion of LC3-I proteins to LC3-II proteins through immunoblotting. However, compared to LC3-I, the LC3-II level was unstable due to its rapid degradation during autophagy by lysosomal proteases. Therefore, to ensure an appropriate interpretation of LC3 immunoblot analysis, specific protease inhibitors must be added before treatments to conserve and prevent the degradation of LC3-II levels. Protease inhibitors, E64-D (Santa Cruz Biotechnology, Dallas, TX) and pepstatin A (Sigma-Aldrich, Oakville, ON), are commonly used to preserve the accumulation of LC3-II. 1mg of E64-D was dissolved 500μL DMSO and 500μL 1X PBS was later added to a final stock concentration of 1mg/mL. 5mg of pepstatin A was dissolved in 100μL glacial acetic acid (Fisher Scientific, Whitby, ON) and 900μL DMSO to a final stock concentration of 5mg/mL. Before treatments, cells were pre-treated with 10μg/mL of E64-D and pepstatin A in standard culture media for 30 minutes. Same concentrations of E64-D and pepstatin A were also added to all treatment conditions.
Fluorescence microscopy

Fluorescence microscopy can be used to visualize fluorescent probe tagged proteins to study autophagic activity of cells. Autophagy was detected using the CYTO-ID® Autophagy detection kit (Enzo Life Sciences, Inc., Farmingdale, NY). This dye specifically stains autophagosomal substrates such as pre-autophagosomal structures, autophagosomes and autophagolysomes that are produced during autophagy. Microscope cover slips (Fisher Scientific, Whitby, ON) were placed in 6-well plates before plating of cells. Seeding, transfection and treatment protocol were as previously described. After treatments, media was aspirated and the cells were washed with 1X PBS. PBS was removed and plates were washed once with assay buffer, consisted of DMEM/high glucose medium without phenol red, supplemented with 10% FBS and 1% L-glutamine (Fisher Scientific, Whitby, ON). The Microscopy Dual Detection Reagent was then prepared by adding 2μL CYTO-ID® Green Detection Reagent and 1μL Hoechst 33342 Nuclear Stain to 500μL assay buffer. 6 x 75μL drops of the Microscopy Dual Detection Reagent were added on a piece of Parafilm M™ wrapping film (Fisher Scientific, Whitby, ON), approximately 8cm x 12.5cm in size. The cover slips were removed from the wells. Cell side down, each coverslip was placed on a Microscopy Dual Detection Reagent droplet. Along with the coverslips, the parafilm was transferred to the incubator for 30 minutes. After incubation, cover glasses were placed back into the wells, cell side up. The coverslips were washed 3 times with assay buffer. 50μL of 1 X PBS was applied onto each of the microscope slides (Fisher Scientific, Whitby, ON). Each coverslip was then placed on the PBS droplet, cell side down, on the microscope slide. The slides were imaged by a fluorescence microscope using standard FITC and DAPI filter sets for autophagic and nuclear signals respectively.

Clonogenic assay

As a measure of the effectiveness of various treatments, clonogenic assasy were performed to determine the capacity of cells to form colonies after treatments. Cells were seeded at 80,000 cells per mL in 12-well plates and incubated for 24 hours. Cells were then transfected with NC1, or PRKARIA
siRNA or left untreated. After 48 hours, cells were treated with corresponding treatment groups for 24 hours. The assay was performed according to the protocol outlined in Franken et al. (Franken et al., 2006). In brief, after treatments, media was aspirated and cells were washed with 1X PBS. The cells were then trypsinized and re-plated into 6-well plates in duplicates with standard culture media at 400 and 800 cells per mL for JL-31 and JL-75 respectively. The fixation solution was prepared by mixing 6% glutaraldehyde and 0.5% crystal violet in deionized water. After 10 – 14 days, media was removed from the wells. The wells were then washed with 1X PBS. The PBS was aspirated and 1.5mL of fixation solution was added into each well. After 2 hours, the fixation solution was removed and the plates were submerged in water. The remaining solution was carefully washed away and the plates were left to dry at room temperature. Colonies were counted manually using a stereomicroscope. A colony was defined to contain a minimum of 50 cells.

**Statistical analysis**

GraphPad Prism 6.0 software (GraphPad Software Inc., CA) was used to conduct multiple t-tests and one or two way analysis of variance (ANOVA). Post-tests were conducted using the Tukey’s multiple comparisons tests. Treatments were considered statistically significant if resulting \( p\)-value was less than 0.05 (\( p<0.05 \)). All graphs were plotted with mean ± standard error of mean (SEM) when applicable.
Results

**Doxorubicin IC<sub>50</sub> of JL-31 and JL-75 OSA cell lines**

LC50 of doxorubicin has been previously described in dogs. However, the susceptibility of doxorubicin could be different in canine OSA cell lines. Therefore it was important to determine the doxorubicin dosages of each OSA cell line needed for treatments. IC<sub>50</sub> is a good indication of the effectiveness of doxorubicin in inhibiting biological functions of these cell lines. Seeding densities of JL-31 and JL-75 in 96-well plates were previously determined to be at 4000 and 5000 cells per well, respectively. Cells were seeded and incubated for 24 hours. Ten serial dilutions of doxorubicin were applied to the wells and the plates were incubated for 24 hours. Metabolic activity was determined by adding the PrestoBlue® Cell Viability solution and read by a plate reader. The data was normalized to wells with media or treatment only. Normalized data was then log-transformed and fitted on a sigmoidal dose-response curve. IC<sub>50</sub> of each cell line was generated by GraphPad Prism. From three biological replicates, the data showed that the IC<sub>50</sub> of doxorubicin of JL-31 and JL-75 were 0.6213μM and 4.187μM respectively (Fig. 3). JL-31 showed a higher susceptibility to doxorubicin than JL-75.

**Quantification of knockdown efficiency**

siRNA-mediated PRKAR1A knockdown was tested in both JL-31 and JL-75 OSA cell lines. In each 12-well plate, each cell line was transfected with 40nM of PRKAR1A siRNA (KD) or NC1, or left untreated (FS). After 48 hours of incubation, cells were harvested and their protein lysates analyzed by western blotting. Western blot analyses showed reduction of R1α expression (49 kDa) in cells transfected with PRKAR1A siRNA when compared to FS and NC1 (Fig. 4). To objectively quantify the transfection efficiency, R1α bands of each treatment were quantified using ImageJ. The intensities of bands were first measured and normalized to the FS band of each cell line. The normalized bands were then adjusted to each of the corresponding β-actin bands, generating an adjusted density. The adjusted densities of each treatment were then plotted on a column graph. T-tests of the adjusted densities of KD groups in both cell lines showed significant reduction in R1α expression levels when compared to FS and NC1 (Fig. 5).
Quantification of autophagic flux

To accurately quantify autophagic flux, LC3-II levels had to be preserved during treatments. To ensure the accumulation of LC3-II levels, lysosomal protease inhibitors (PI), E-64D and pepstatin A were applied to the cells before treatment. However, it was important to observe the differences between non-PI and PI-treated groups in LC3-I and LC3-II levels and whether the PIs would interfere with the expression levels of p62, which recognizes autophagic cargoes. Therefore, a comparison study was done by treating JL-31 and JL-75 OSA cells with IC₅₀ of doxorubicin, using FS and SF (serum free) as controls, with or without PI. Cells were seeded at 80,000 cells per mL and incubated for 24 hours. PI-treated groups were pre-treated with standard culture media supplemented with E64-D (10μg/mL) and pepstatin A (10μg/mL) for 30 minutes. After the pre-treatments, cells were washed with 1 X PBS and treated with respective conditions. SF groups were cultured in media without the supplementation of FBS and L-glut. DOXO groups were treated with IC₅₀ of doxorubicin of JL-31 and JL-75 (0.62μM and 4.21μM). PI-treated groups were cultured with E-64D and pepstatin A along with treatment conditions. Cells were treated for 24 hours before being harvested and analyzed by western blotting. The intensities of LC3-II band of both PI-treated cells were denser than the non-PI treated cells (Fig. 6A,B). In both cell lines, doxorubicin-treated groups had increased LC3-II levels when compared to FS groups (Fig. 6A,B). In JL-31, although LC3-II levels were elevated in SF groups, LC3-I levels were increased as well (Fig. 6A). This trend was not found in JL-75, similar to the doxorubicin-treated groups, SF groups exhibited reduced LC3-I levels and increased LC3-II levels in both non-PI and PI-treated groups (Fig. 6B). p62 expression levels were similar in both non-PI and PI-treated groups (Fig. 6A,B). p62 levels in both SF and doxorubicin-treated groups were lower than FS, and doxorubicin-treated groups had the lowest p62 levels among all 3 treatments. The lower p62 levels in doxorubicin-treated groups indicated that doxorubicin was more effective in inducing autophagy in both cell lines.

To assess the autophagic flux between the non-PI and PI-treated groups, LC3-I and LC3-II bands were quantified, normalized and adjusted as described above. As LC3-II is more sensitive in
immunoblotting, the autophagic flux was a ratio calculated by the adjusted density of LC3-II / (LC3-I + LC3-II). This method takes the total LC3 protein into account and generates a ratio of LC3-II relative to the conversion of LC3-I to LC3-II. After the quantification, the data was plotted on bar graphs (Fig. 7). In JL-31, the quantification showed similar autophagic flux between non-PI and PI-treated groups (Fig. 7). Doxorubicin-treated groups had the highest autophagic flux when compared to both FS and SF groups (Fig. 7). In JL-75, non-PI and PI-treated groups showed similar trends in autophagic flux (Fig. 7). However, the separation of values between FS and SF or DOXO was greater in PI-treated groups than non-PI treated groups (Fig. 7). Both SF and DOXO showed higher autophagic flux than FS in non-PI and PI groups, but different from JL-31 cells, DOXO was not better than SF (Fig. 7).

Determining concentrations of autophagy modulators required for treatments

Rapamycin and L690, 330 were chosen to be used as positive and HCQ as a negative control. The concentrations of treatments were determined by various published studies. Both rapamycin and L690, 330 induce autophagic response. While rapamycin induces autophagy in a mTOR0dependent way, L690, 330 induces autophagy in a mTOR-independent manner. HCQ inhibits autophagy by elevating the pH level in autophagylsosomes and prevents degradation. Cells were seeded at 80,000 cells per mL in 12-well plates and incubated for 24 hours. Cells were pre-treated with E-64D (10μg/mL) and pepstatin A (10μg/mL) in standard culture media for 30 minutes. After the pre-treatment, 4 different concentrations of rapamycin and L690, 330 were applied to the cells, supplemented with E-64D and pepstatin A. HCQ treatments groups were treated in serum free media supplemented with the same concentrations of PI. After 24 hours, Cells were then harvested and analyzed by western blotting.

In both JL-31 and JL-75, all rapamycin-treated groups had elevated LC3-II and reduced LC3-I level when compared to the FS groups (Fig. 8). Based on this result, 100nM rapamycin was selected to be used as positive control for both JL-31 and JL-75 in subsequent experiments due to its low concentration and effectiveness in inducing autophagy. For HCQ, in JL-31, LC3-II levels of all 4 HCQ-treated samples were visibly higher than both FS and SF (Fig. 9). LC3-I levels of all 4 HCQ-treated samples were higher
than SF, with 1μM and 20μM samples having the highest LC3-I levels (Fig. 9). In JL-75, all HCQ-treated samples had increased accumulation of LC3-II except at 20μM (Fig. 9). Both 20μM and 50μM had small increase in LC3-I levels. The increased accumulation of LC3-II protein in all treatment groups was due to the inhibition of degradation of autophagolysosomes. By increasing the pH level of autophagylysosomes, lysosomes were not able to complete the degradation. The extreme accumulation of LC3-II protein in treatment groups prevented accurate quantification through densitometry. 20μM was selected to be the treatment concentration due to its comparability to other in vitro studies. In L690, 330 treatments, in JL-31, LC3-II levels were elevated in 200μM and 400μM (Fig. 10). 400μM had the highest LC3-II level, comparable to rapamycin (Fig. 10). In JL-75, there was consistent dosage-dependent increase in LC3-I and II levels across L690, 330 treatments (Fig. 10). 400μM was selected to be used as treatment concentration in subsequent experiments as it showed sufficient induction of autophagy in both cell lines (Fig. 10).

*Autophagy in transfected OSA cell lines under treatments*

After all the treatment conditions were determined, the differences in autophagy between PRKARIA KD and control cells could be investigated. Cells were seeded at 80,000 cells per mL in 12-plates for 24 hours. After 24 hours, cells were either left untreated or transfected with PRKARIA siRNA or NC1 for 48 hours. Cells were then pre-treated with E-64D (10μg/mL) and pepstatin A (10μg/mL) in standard culture media for 30 minutes. After pre-treatments, cells were treated with DOXO IC50 and/or 400μM L690 (referred from now on as L690), supplemented with E-64D and pepstatin A for 24 hours. Cells were then harvested and subjected to western blotting. In both JL-31 and JL-75, all doxorubicin-treated groups had reduced p62 expression levels relative to non-doxorubicin treated groups (fig. 11, 12). In JL-31, the NC1 group treated with doxorubicin and L690 had the lowest p62 expression level among all treatment groups (Fig. 11). In JL-75, NC1 groups treated with doxorubicin and combination treatment of doxorubicin and L690 had lower p62 expression levels than the KD groups under the same treatments (Fig. 12). R1α expression levels indicated sufficient PRKARIA KD in all cell lines (fig 11, 12).
Regarding LC3 levels, in the non-treatment groups of JL-31, both FS and NC1 showed high LC3-I level and low LC3-II level, while KD showed lower LC3-I level and slight increase in LC3-II level (Fig. 11). Quantification of these bands showed elevated autophagic flux in KD when compared to FS and NC1 (Fig. 13). In the doxorubicin-treated groups, NC1 showed higher LC3-II level and lower LC3-I level than KD (Fig. 13). Quantification of these bands showed doxorubicin-treated NC1 had a higher autophagic flux than KD (fig. 13). A similar trend was also seen in the combination treatments groups and the L690, 330 only groups, where NC1 had higher LC3-II levels and reduced LC3-I levels compared to KD (Fig. 13). Quantification also showed higher autophagic flux and NC1 groups than KD groups (Fig. 13). All treatment groups had higher autophagic flux than the non-treated groups (Fig. 13). In JL-75, all non-treated groups had higher LC3-I levels than LC3-II. However, NC1 showed the highest LC3-I and LC3-II expression levels when compared to FS and KD (Fig. 12). Quantification of LC3-I and LC3-II bands showed KD had the highest autophagic flux when compared to FS and NC1 (Fig. 13). In the doxorubicin-treated group, NC1 had a higher LC3-II expression level and lower LC3-I level than KD (Fig. 12). In the combination treatment groups, again, NC1 had the higher LC3-II expression level and lower LC3-I level than KD. When compared to the doxorubicin-treated group, the intensity of LC3-II band of NC1 was visibly higher in the combination treatment group (Fig. 12). In the L690 only groups, NC1 had a higher LC3-II expression level than KD. Quantification of all treatment groups showed NC1 had higher autophagic flux in all treatments when compared to KD (Fig. 13).

**Clonogenic survival assay**

To compare the clinical effectiveness of these treatments within different transfection groups, clonogenic survival assays were performed on all treatment groups as specified in the Materials and Methods section. Since different cell lines have dissimilar plating efficiencies (PE), colony data had to be normalized to the colony number of untreated cells of that specific cell line (Franken et al., 2006). PE was the ratio of number of colonies formed divided by number of cells seeded. Number of colonies formed
after treatments, normalized by PE is the surviving fraction. Survival fraction is calculated by the number of colonies formed after treatment divided by number of cells seeded multiply by PE.

Survival fractions of NC1 and KD in the same treatment group were plotted beside each other on bar graphs. In JL-31, doxorubicin-treated groups had lower surviving fraction than the non-doxorubicin treated groups (fig 14). However, multiple t-test analyses showed that there was no statistical significance between NC1 and KD across all treatments (Table 1). Statistical significance was also not found when survival fractions of doxorubicin-treated FS, NC1 and KD were analyzed using 1way ANOVA (Table 2). In JL-75, there was no discernible difference in survival fractions between NC1 and KD across all treatments (Fig. 14). Multiple t-tests also showed no statistical significance between NC1 and KD in all treatments (Table 1). Doxorubicin-treated groups also showed no statistical significance when analyzed by 1way ANOVA (Table 3). Within the same transfection group, surviving fractions of different treatment groups were then compared and analyzed by two way ANOVA. No statistical significance was achieved between all treatments and transfection conditions in both cell lines (Table 4, 5).
Figure 3: Sigmoidal dose-response curves of JL-31 and JL-75 OSA cells under serial dilutions of doxorubicin. Cells were seeded in 96-well plates at 4000 cells and 5000 cells for JL-31 and 75 respectively. After 24 hours, cells were treated with 10 serial dilutions, from 0.05μM to 100μM, for 72 hours. Cell viability was then detected using the PrestoBlue® Cell Viability solution and read by a plate reader. Cell viability data was normalized by subtracting the mean fluorescence measurements of treatments from fluorescence measurements of wells with media or treatment only. Log transformations were performed on the dosages (X-axis values) to establish sigmoidal dose-response curves. Data shown here are average means of 3 biological replicates with SEM error bars.
Figure 4: Comparisons of different levels of R1α expression in full serum (FS, untreated), scrambled siRNA negative control (NC1) and PRKARIA siRNA knockdown (KD) in JL-31 and JL-75 OSA cells. Cells were seeded in a 6-well plate at 8 x 10^4 cells per mL for both JL-31 and 75. After 24 hours, cells were either left untreated or transfected using Lipofectamine 3000 with corresponding siRNA and incubated for 48 hours. Western blot analyses showed significant reduction of R1α expressions (49 kDa) in cells transfected with PRKARIA siRNA. β-actin (42 kDa) expressions were used to ensure uniform protein loading.

Figure 5: Bar graphs of transfection efficiency of JL-31 and JL-75 OSA cells. Cells were seeded in 12-well plates at 8 x 10^4 cells per mL and incubated for 24 hours. Cells were then either left untreated or transfected with corresponding siRNA. Cells were harvested after 48 hours. Western blot analyses were done on the harvested protein samples, targeting R1α and β-actin as loading control. The densities of bands of R1α were quantified using ImageJ. Each band was normalized to the full serum (FS, untreated) band on the same blot and adjusted to the corresponding load-control protein band. The experiment was run in triplicate. Data shown here are average means and SEM error bars. R1α expression levels of KD groups in both cell lines showed statistically significant reduction when compared to the untreated and NC1. ** and *** indicate statistical significance (p<0.01 and p<0.001), respectively.
Figure 6: Expression levels of LC3-I, LC3-II and p62 during full serum (FS), serum free (SF) and doxorubicin (DOXO), with or without lysosomal protease inhibitors (PI) treatments in JL-31 and JL-75 OSA cells. Cells were seeded as previously described. After the PI pre-treatments, cells were treated with respective conditions. SF groups were cultured in media without the supplementation of FBS and L-glut. DOXO groups were treated with IC₅₀ of doxorubicin of JL-31 and JL-75 (0.62μM and 4.21μM). PI-treated groups were cultured with E-64D and pepstatin A along with treatment conditions. Cells were treated for 24 hours before being harvested. Western blot analyses were done targeting LC3-I and II, p62 and β-actin as loading control.
Differences of autophagic flux in JL-31 OSA cells induced by PI-treatments

Differences of autophagic flux in JL-75 OSA cells induced by PI-treatments

Figure 7: Bar graphs of autophagic flux in JL-31 and JL-75 OSA cells with or without lysosomal protease inhibitors in full serum, serum free and doxorubicin treatments. Intensities of bands of figure 4 were quantified using ImageJ. Data was adjusted to the FS group of each condition and normalized to the β-actin band of each treatment.
Figure 8: Expression levels of LC3-I and LC3-II of JL-31 and JL-75 OSA cells treated with four concentrations of rapamycin. Cells were seeded as previously described. Cells were pre-treated with E-64D (10μg/mL) and pepstatin A (10μg/mL) in standard culture media for 30 minutes. Cells were then treated with 4 different concentrations of rapamycin, supplemented with E-64D and pepstatin A for 24 hours. Full serum (FS) and serum free (SF) were used as controls. Cells were then harvested and analyzed by western blot, targeting the expression levels of LC3-I, LC3-II and β-actin as loading control.
Figure 9: Expression levels of LC3-I and LC3-II of JL-31 and JL-75 OSA cells treated with four concentrations of hydroxychloroquine (HCQ). Cells were seeded as previously described. Cells were pre-treated with E-64D (10μg/mL) and pepstatin A (10μg/mL) in standard culture media for 30 minutes. Cells were then treated with 4 different concentrations of HCQ in serum free media, supplemented with E-64D and pepstatin A for 24 hours. Full serum (FS) and serum free (SF) were used as controls. Cells were then harvested and analyzed by western blot, targeting the expression levels of LC3-I, LC3-II and β-actin as loading control.
Figure 10: Expression levels of LC3-I and LC3-II of JL-31 and JL-75 OSA cells treated with four concentrations of IMPase inhibitor L-690,330. Cells were seeded as previously described. Cells were pre-treated with E-64D (10μg/mL) and pepstatin A (10μg/mL) in standard culture media for 30 minutes. Cells were then treated with 4 different concentrations of L-690, 330 in standard culture media, supplemented with E-64D and pepstatin A for 24 hours. Full serum (FS) was used as negative control while serum free (SF) and 100nM rapamycin (RAP) were used as positive controls. Cells were then harvested and subjected to western blot analysis, to access the expression levels of LC3-I, LC3-II and β-actin serving as loading control.
Figure 11: Expression levels of LC3-I, LC3-II, R1α and p62 in transfected JL-31 OSA cells under doxorubicin (DOXO), L690, 330 (L690) or both treatments combined. Cells were seeded as previously described. After 24 hours, cells were either left untreated or transfected with NC1 or PRKAR1A siRNA for 48 hours. Cells were then pre-treated with E-64D (10μg/mL) and pepstatin A (10μg/mL) in standard culture media for 30 minutes. After pre-treatments, cells were treated with DOXO IC50 and/or 400μM L690, supplemented with E-64D and pepstatin A for 24 hours. Cells were then harvested and subjected to western blot analysis of LC3-I and II, p62, R1α and β-actin as loading control.
Figure 12: Expression levels of LC3-I, LC3-II, R1α and p62 in transfected JL-75 OSA cells under doxorubicin (DOXO), L690, 330 (L690) or both treatments. Cells were seeded as previously described. After 24 hours, cells were either left untreated or transfected with NC1 or PRKAR1A siRNA for 48 hours. Cells were then pre-treated with E-64D (10μg/mL) and pepstatin A (10μg/mL) in standard culture media for 30 minutes. After pre-treatments, cells were treated with DOXO IC₅₀ and/or 400μM L690, supplemented with E-64D and pepstatin A for 24 hours. Cells were then harvested and subjected to western blot analysis of LC3-I and II, p62, R1α and β-actin as loading control.
Figure 13: Autophagic flux in transfected JL-31 and JL-75 OSA cells under doxorubicin (DOXO), L690, 330 (L690) or both treatments. Quantification of LC3 Bands of figure 9 and 10. Autophagic flux was calculated by dividing the intensity of LC3-II band by the total LC3 expression level (LC3-I + LC3II).
Table 1: Multiple T-tests of treatments of clonogenic survival assay

<table>
<thead>
<tr>
<th>Treatments</th>
<th>NC1</th>
<th>KD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment</td>
<td>1.161 ± 0.053</td>
<td>1.026 ± 0.043</td>
<td>0.298</td>
</tr>
<tr>
<td>DOXO</td>
<td>0.377 ± 0.126</td>
<td>0.479 ± 0.073</td>
<td>0.430</td>
</tr>
<tr>
<td>L690+DOXO</td>
<td>0.544 ± 0.029</td>
<td>0.402 ± 0.086</td>
<td>0.272</td>
</tr>
<tr>
<td>L690</td>
<td>0.989 ± 0.040</td>
<td>0.871 ± 0.165</td>
<td>0.361</td>
</tr>
<tr>
<td>HCQ+DOXO</td>
<td>0.389 ± 0.056</td>
<td>0.435 ± 0.120</td>
<td>0.716</td>
</tr>
<tr>
<td>L690+HCQ+DOXO</td>
<td>0.455 ± 0.091</td>
<td>0.613 ± 0.09</td>
<td>0.223</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatments</th>
<th>NC1</th>
<th>KD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment</td>
<td>1.193 ± 0.194</td>
<td>0.934 ± 0.053</td>
<td>0.081</td>
</tr>
<tr>
<td>DOXO</td>
<td>0.908 ± 0.109</td>
<td>0.780 ± 0.059</td>
<td>0.376</td>
</tr>
<tr>
<td>L690+DOXO</td>
<td>0.892 ± 0.187</td>
<td>0.767 ± 0.052</td>
<td>0.387</td>
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<tr>
<td>L690</td>
<td>0.912 ± 0.245</td>
<td>0.910 ± 0.164</td>
<td>0.989</td>
</tr>
<tr>
<td>HCQ+DOXO</td>
<td>0.650 ± 0.175</td>
<td>0.573 ± 0.212</td>
<td>0.594</td>
</tr>
<tr>
<td>L690+HCQ+DOXO</td>
<td>0.514 ± 0.222</td>
<td>0.578 ± 0.243</td>
<td>0.656</td>
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Statistical analyses of clonogenic survival assay results of treatments of JL-31 and JL-75 OSA cells. Multiple t-tests were performed on means of the two transfection conditions within the same treatment and cell line.
Figure 15: Bar graph of clonogenic survival assays of JL-31 OSA cells after doxorubicin treatment. Cells were seeded as previously and treated as described with an added full serum (FS, untreated) control. After 24 hours, cells were transfected with either NC1 or PRKAR1A siRNA (KD). After 48 hours, cells were treated with doxorubicin for 24 hours. Once treatments were completed, cells were trypsinized and re-suspended at 400 cells per mL. Cells were then seeded in 6-wells plates for 10 – 14 days. After the proliferation period, cells were stained with fixation solution, consisting of 6% glutaraldehyde, 0.5% crystal violet and water. Colonies were counted manually using a stereomicroscope. The experiment was run in triplicate. Data shown here are average of means and SEM error bars.

Table 2: 1 way ANOVA of clonogenic survival post-doxorubicin of JL-31

<table>
<thead>
<tr>
<th>Treatments</th>
<th>FS</th>
<th>NC1</th>
<th>KD</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.290 ± 0.125</td>
<td>0.377 ± 0.126</td>
<td>0.479 ± 0.073</td>
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ANOVA table

<table>
<thead>
<tr>
<th></th>
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<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Treatment (between columns)</td>
<td>0.0537</td>
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<td>0.027</td>
<td>F (2, 6) = 0.730</td>
<td>0.520</td>
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<tr>
<td>Residual (within columns)</td>
<td>0.221</td>
<td>6</td>
<td>0.037</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.275</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Statistical analysis of clonogenic survival data of JL-31 cells after doxorubicin treatment. No statistical significance (p<0.05) was found across all conditions.
Figure 16: Bar graph of clonogenic survival assays of JL-75 OSA cells after doxorubicin treatment. Cells were seeded as previously and treated as described with an added full serum (FS, untreated) control. After 24 hours, cells were transfected with either NC1 or PRKAR1A siRNA (KD). After 48 hours, cells were treated with doxorubicin for 24 hours. Once treatments were completed, cells were trypsinized and re-suspended at 800 cells per mL. Cells were then seeded in 6-wells plates for 10 – 14 days. After the proliferation period, cells were stained with fixation solution, consisting of 6% glutaraldehyde, 0.5% crystal violet and water. Colonies were counted manually using a stereomicroscope. The experiment was run in triplicate. Data shown here are average of means and SEM error bars.

Table 3: 1 way ANOVA of clonogenic survival post-doxorubicin of JL-75

<table>
<thead>
<tr>
<th>Treatments</th>
<th>FS</th>
<th>NC1</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.760 ± 0.026</td>
<td>0.908 ± 0.109</td>
<td>0.780 ± 0.059</td>
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</table>

<table>
<thead>
<tr>
<th>ANOVA table</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (between columns)</td>
<td>0.039</td>
<td>2</td>
<td>0.019</td>
<td>F (2, 6) = 0.122</td>
<td>0.360</td>
</tr>
<tr>
<td>Residual (within columns)</td>
<td>0.096</td>
<td>6</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.135</td>
<td>8</td>
<td>0.016</td>
<td></td>
<td></td>
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Statistical analysis of clonogenic survival data of JL-75 cells after doxorubicin treatment. No statistical significance (p<0.05) was found across all conditions.
Table 4: 2 way ANOVA of clonogenic survival post-treatments of JL-31

<table>
<thead>
<tr>
<th>Treatments</th>
<th>NC1</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Difference</td>
<td>95% CI of diff.</td>
</tr>
<tr>
<td>DOXO vs. L690+DOXO</td>
<td>-0.167</td>
<td>-0.516 to 0.181</td>
</tr>
<tr>
<td>DOXO vs. HCQ+DOXO</td>
<td>-0.011</td>
<td>-0.360 to 0.337</td>
</tr>
<tr>
<td>DOXO vs. L690+HCQ+DOXO</td>
<td>-0.077</td>
<td>-0.426 to 0.271</td>
</tr>
<tr>
<td>L690+DOXO vs. HCQ+DOXO</td>
<td>0.156</td>
<td>-0.193 to 0.504</td>
</tr>
<tr>
<td>L690+DOXO vs. L690+HCQ+DOXO</td>
<td>0.090</td>
<td>-0.259 to 0.438</td>
</tr>
<tr>
<td>HCQ+DOXO vs. L690+HCQ+DOXO</td>
<td>-0.066</td>
<td>-0.414 to 0.283</td>
</tr>
</tbody>
</table>

Statistical analyses of clonogenic survival data of by comparing different treatments approaches with respect to transfection conditions. No statistical significance (p<0.05) was found across all treatment and transfection conditions.

Table 5: 2 way ANOVA of clonogenic survival post-treatments of JL-75

<table>
<thead>
<tr>
<th>Treatments</th>
<th>NC1</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Difference</td>
<td>95% CI of diff.</td>
</tr>
<tr>
<td>DOXO vs. L690+DOXO</td>
<td>-0.02</td>
<td>-0.781 to 0.742</td>
</tr>
<tr>
<td>DOXO vs. HCQ+DOXO</td>
<td>0.243</td>
<td>-0.519 to 1.00</td>
</tr>
<tr>
<td>DOXO vs. L690+HCQ+DOXO</td>
<td>0.379</td>
<td>-0.383 to 1.14</td>
</tr>
<tr>
<td>L690+DOXO vs. HCQ+DOXO</td>
<td>0.262</td>
<td>-0.500 to 1.02</td>
</tr>
<tr>
<td>L690+DOXO vs. L690+HCQ+DOXO</td>
<td>0.398</td>
<td>-0.363 to 1.160</td>
</tr>
<tr>
<td>HCQ+DOXO vs. L690+HCQ+DOXO</td>
<td>0.136</td>
<td>-0.626 to 0.898</td>
</tr>
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</table>

Statistical analyses of clonogenic survival data of by comparing different treatments approaches with respect to transfection conditions. No statistical significance (p<0.05) was found across all treatment and transfection conditions.
Discussion

Doxorubicin: Selection, IC$_{50}$ and defining cell viability

Investigations of autophagy in JL-31 and JL-75 OSA cells started with the selection of the main chemotherapy agent. While carboplatin and cisplatin are also commonly used to treat canine OSA, doxorubicin was chosen due to its susceptibility of the OSA cell lines to this chemotherapy agent. In the work of a previous graduate student at our laboratory, while both JL-31 and JL-75 cells responded well to doxorubicin, it was shown that these OSA cells could tolerate carboplatin well and the determination of IC$_{50}$ was problematic. Once the chemotherapy agent was selected, measurements of IC$_{50}$ of doxorubicin could then proceed.

Originally, serial dilutions of doxorubicin from 10μM to 0.001μM were used to treat both OSA cell lines. However, treatment concentrations had to be altered due to the finding that both cell lines experienced elevated metabolic activities at lower dosages (0.05μM - 0.001μM) (data not shown). Although both cell lines originated from different patients, since these cell lines proliferated under light doxorubicin-stress, it was speculated that these cells possess a certain level of chemo-resistance. For JL-31, this resistance could likely be acquired due to previous exposure to chemotherapy while JL-75 could have an intrinsic resistance to doxorubicin as chemotherapy was not administered to the host at the time of amputation. Therefore, in order to generate an applicable dose-response curve, 0.05μM, instead of media only, was chosen as the starting dosage in which the cell metabolic activity was the highest in both cell lines.

Using serial dilutions of doxorubicin, an IC$_{50}$ for both cell lines was determined using a dose-response curve (Fig. 3). It was determined that the doxorubicin IC$_{50}$ of JL-31 and JL-75 was 0.62μM and 4.19μM respectively. Immediately, we noticed the vast difference in IC$_{50}$ in the cell lines, with JL-75’s IC$_{50}$ exceeding the LD$_{50}$ for dogs (2kg/mL, 3.45μM) (Lewis, 2004). Moreover, metastatic OSA cells were notoriously aggressive even after chemotherapy (Wycislo & Fan, 2015). Therefore, it is logical to predict that JL-31 would require a higher doxorubicin dosage to achieve IC$_{50}$ when compared to JL-75, a
primary OSA cell line. This discrepancy could be explained by two main reasons. Firstly, JL-31 could be sensitized to chemotherapy intervention. Even though the cells showed resistance towards low-dosages of doxorubicin (data not shown), a higher concentration of doxorubicin could lead to an elevated apoptotic response. This was investigated in a study by Gonçalves et al. in which the authors discovered that OSA cancer stem-like cells (CSCs) were more likely to go into apoptosis after exposure to doxorubicin (Gonçalves et al., 2015).

The second possible reason for this discrepancy is the method of how IC₅₀ was determined. IC₅₀ was determined by performing a cell viability assay in which a resazurin-based solution was added to treatment wells. Resazurin, while naturally blue, is reduced in metabolically active mitochondria into resorufin, a pink and highly fluorescent product. Therefore, resazurin’s dependence on metabolic activity for detection of the fluorescent product means that it cannot always differentiate between live or dead cells. It is possible that a population of JL-31 cells became dormant (metabolically inactive, but still viable) under doxorubicin treatment. Clinically undetectable dormant micrometastases are highly prevalent in canine OSA (Fenger et al., 2014). It is possible that in our study, the cells were metabolically inactive but alive and this was not detected using a resazurin-based assay.

An alternative to cell viability assay is to quantify live and dead cells using flow cytometry with the 7-AAD fluorescent dye. Dead cells often have compromised cell membrane and 7-AAD marks the DNA, especially in GC regions. This approach could give us a better estimation of live and dead cells and could generate more accurate IC₅₀ dosages.

siRNA-mediated PRKAR1A knockdown: R1α expression level and transfection efficiency

PRKAR1A siRNA was previously designed by our graduate student and transfections were performed using Lipofectamine 3000, according to manufacturer’s protocol. To confirm the effectiveness of the KD, expression levels of R1α were detected by western blot analysis (Fig. 4). To further quantify the transfection efficiencies of these cells, densitometries were performed for R1α bands of two replicates
(Fig. 5). In figure 4, the R1α expression levels of both JL-31 and JL-75 were lower in the KD groups when compared to FS and NC1. With a lowered level of R1α, the bands indicated that the KD was successful (Fig. 4). Quantification also showed >70% reduction in R1α expression levels in KD groups compared to FS and NC1 (Fig. 5). This experiment ensured the current aliquots of siRNA were still functioning effectively as siRNA is prone to degradation overtime.

Interestingly, R1α expression levels in NC1 were shown to be elevated in both western blot (Fig. 4) and densitometry (Fig. 5). According to manufacturer’s description, the siRNA of NC1 does not recognize any sequences in human, mouse or rats. Although highly improbable, since NC1 was not tested in canines, it could potentially induce an off-target effect that elevated the R1α expression levels of NC1-treated cells.

Lipofectamine is a common transfection vehicle for mRNA and siRNA. When performed correctly, this liposome approach can transfet siRNA effectively and efficiently while maintaining a low toxicity to cells (T. K. Kim & Eberwine, 2010). However, this approach is cumbersome as siRNA has to be transfected for each experiment and therefore prone to errors and inconsistencies. Moreover, ideally there should also be a group where PRKAR1A is overexpressed in order to make clinical comparisons. Although time consuming, new technologies like the CRISPR (Clustered regularly interspaced short palindromic repeats)-Cas9 system could enable us to modify transcriptional activation and/or inactivation of PRKAR1A and generate overexpressed and down-regulated colonies, using our current canine OSA cell lines (Graham & Root, 2015).

Examination of autophagic flux: Selection of lysosomal protease inhibitors, rationale and quantification

Autophagy is a highly complex and dynamic process in which cellular products are subjected to proteolytic degradation. Measuring autophagy has proven to be a taunting task; mainly due to the fact that autophagic substrates and products only exist for a finite amount of time and there is a lack of the means to produce a reliable and quantitative approach to monitor autophagy as it is occurring (Barth et al.,
Although autophagy was first characterized by transmission electron microscopy (TEM), this approach is not quantitative, and the lack of expertise, time and the large number of necessary treatments prohibited us from using this approach.

Currently, the most popular way of analyzing autophagy is through western blotting by measuring microtubule-associated protein 1 light chain 3 (LC3) protein and p62. LC3 exists in 2 forms; the cytosolic form (LC3-I) and lipided form (LC3-II). Upon autophagy activation, with the facilitation of Atgs, LC3-I is conjugated to the lipophilic phosphatidylethanolamine (PE) to produce LC3-II (Nakatogawa et al., 2009). PE also assists with the integration of LC3-II at pre-autophagosomal substrates and autophagosomes. On the other hand, p62 has been shown to support the selection of damaged proteins that are tagged with ubiquitin (Wurzer et al., 2015). Through the interaction with LC3-II, p62 links the damaged proteins to the LC3-II for degradation (Wurzer et al., 2015). Together, these proteins are degraded once lysosomes are fused with autophagosomes, forming autophagolysosomes. Therefore, western blot targeting LC3-I, II and p62 provide us with means to monitor autophagy.

However, western blot for these proteins is not as straightforward as for other proteins. Although p62 levels change during autophagy and its degradation could indicate an autophagic response, it is also regulated by the RAS oncogene and NF-κB activity and is therefore not completely specific for autophagy (Barth et al., 2010). Previous studies have shown that LC3-II has a higher affinity for anti-LC3 antibodies than LC3-I (Mizushima & Yoshimori, 2007). Moreover, LC3-I and II levels are cell specific; different cell lines might express a certain level of LC3-I and LC3-II during normal conditions (D. Klionsky et al., 2016). Therefore, data generated by solely comparing the intensity ration between LC3-I and II could potentially be an inaccurate representation of autophagy (Barth et al., 2010). Furthermore, determining changes in LC3-II could be challenging as it is degraded along with autophagosomes by lysosomes(Mizushima, 2007). To better quantify the changes of LC3-II expression levels, it was our priority to select an approach that could prevent the degradation of LC3-II.
There were three main approaches that we considered to facilitate with the accumulation of LC3-II; V-ATPase inhibition, neutralization of lysosomal pH and inhibition of lysosomal protease (Barth et al., 2010; Mizushima, 2007; Nair et al., 2012).

Lysosomal proton pump V-ATPases promote proton translocation and in turn lower the pH level of the organelle lumen. The decrease in pH level is crucial for the activation of lysosomal hydrolases and degradation of various cargo (Mauvezin et al., 2015). V-ATPase inhibitors such as Bafilomycin A1, increase lysosomal pH, and thus prevents the fusion of autophagosomes with lysosomes and preserve the expression of LC3-II (D. Klionsky et al., 2016). However, the results of blockage of autophagosome-fusion through Bafilomycin A1 have been confusing (D. J. Klionsky et al., 2008; Y. Yang et al., 2013). It was speculated that extended treatment time required for Bafilomycin A1 could interfere with experimental results due to cytotoxicity and disruption of mitochondria (D. Klionsky et al., 2016). There is also a concern that the use of Bafilomycin A1 could be a confounding factor as the reduction of lysosomal amino acids could deactivate mTOR activity, leading to an activation of autophagy (Juhász, 2012). Due to these factors and the fact that our experimental timeframe for treatments was 24 hours, Bafilomycin A was not an ideal candidate.

Another approach that we considered was the neutralization of lysosomal pH by lysosomal lumen alkalizers, such as HCQ. By neutralizing the pH of lysosomes, autophagy is impaired and LC3-II could accumulate (Y. Yang et al., 2013). However, since HCQ was later used as part of our treatment protocol as an autophagy inhibitor, it was not further considered.

Lysosome proteases consist of three main types; cysteine, aspartic acid and serine proteases (D. Klionsky et al., 2016). Using protease inhibitors (PI) such as E-64D (aspartic), pepstatin A (cysteine and serine) and leupeptin (threonine) could prevent lysosomal degradation through the inhibition of cathepsins (Y. Yang et al., 2013). Lysosomal cathepsins are proteases that are activated in low pH condition and assist with the degradation of autophagic substrates (Y. Yang et al., 2013). However, since there are
different subtypes of cathepsins, it is a common practice to utilize multiple lysosome protease inhibitors. E-64D and pepstatin A are often used together to inhibit a range of cathepsins subtypes. This is particularly attractive for us, and ultimately our main reason for choosing this approach as these lysosomal protease inhibitors selectively target a subset of proteases, while other approaches like V-ATPase inhibitors and HCQ interfere with all lysosomal acid hydrolases (Seguin et al., 2014). One major problem with the utilization of pepstatin A was the relatively long incubation time required to exert its inhibitory effect (D. Klionsky et al., 2016). This did not present a problem for our experiments as the treatment protocol was 24 hours.

To confirm the effectiveness of our chosen lysosomal protease inhibitors, we performed two identical experiments with one group subjected to PI-treatments. For this experiment, cells were pre-treated with E-64D and pepstatin A for 30 minutes prior to treatments. These protease inhibitors were also added to the treatments to ensure sufficient accumulation of LC3-II. From figure 6, we noticed western blot bands of LC3-II in the PI-treated groups were more intense than the non-PI treated groups in both cell lines. Interestingly, when compared to the doxorubicin group, JL-31 was shown to be more resistant to conversion of LC3-I to LC3-II during SF condition (Fig. 6). This result was in agreement with the p62 expression level, which did not experience any significance reduction when compared to the p62 level of doxorubicin (Fig. 6). This data is particularly important as it indicates that SF conditions are not sufficient to induce autophagy in JL-31 cells, suggesting that these cells are resistant to environmental stress and might not be an appropriate positive control for autophagy.

True assessment of autophagy activity is through the determination of autophagic flux, in which the conversion of LC3-I and LC3-II is quantitatively measured. By preserving LC3-II, we could compare conversion ratio of LC3 proteins of different treatments within the same cell line by dividing LC3-II by LC3-I + LC3-II (Mizushima & Yoshimori, 2007). The intensities of bands were adjusted to the FS group and normalized using each protein’s sample’s β-actin loading control band. Figure 7 was the result of quantification of figure 6. In JL-31, doxorubicin-treated group exhibited the highest autophagic flux while
SF displayed a slight elevation in autophagic flux when compared to FS in both non-PI and PI-treated groups (Fig. 7). In contrast, by a small margin, SF was shown to induce a higher autophagic flux than the doxorubicin-treated group, when compared to FS in JL-75 in both non-PI and PI-treated groups (Fig. 7). Interestingly, while exhibiting the same trend, the separation of autophagic flux between FS and SF or doxorubicin was higher in the PI-treated group (Fig. 7). This was the result of protease inhibitors treatment. By preserving the accumulation of LC3-II protein, western blot bands were more intense and therefore the quantification of autophagic flux could be more sensitive. It is also very important to note that the autophagic flux value could only be used to compare the relative differences in autophagy between different treatments within the same cell line as this value is by no means a physiological value of autophagy.

**Autophagy modulators: Rapamycin, Hydroxychloroquine and L690, 330**

Establishing true positive and negative controls of autophagy was crucial to our experiments because of the innate differences in autophagic response between cell lines and to validate our current western blot assays. Rapamycin was chosen as the positive control due to its effectiveness in inducing autophagy through inhibition of mTOR. Rapamycin elicits its effect by binding to FKBP12 (FK506-binding protein of 12 kDa) which complexes and inhibits mTORC1 (Renna et al., 2010). HCQ was chosen because of its clinical relevance. HCQ is commonly used as an antimalarial drug and in autoimmune diseases such as rheumatoid arthritis and lupus (Ben-Zvi et al., 2012). As described above, HCQ inhibits autophagy by inhibiting lysosomal hydrolase activities through lysosome alkalization (Z. J. Yang et al., 2011). Several studies have utilized HCQ to augment cytotoxicity of chemotherapy agents by inhibition of autophagy (Rangwala et al., 2014; Sui et al., 2013). In addition to these controls, since it was speculated that down-regulation of PRKAR1A could lead to an mTOR dependent autophagy inhibition, the effect could potentially be rescued by mTOR independent autophagy activation. Lithium chloride (LiCl) was originally chosen for this purpose. LiCl can induce autophagy through the inhibition of inositol monophosphataes (IMPase) and reduce intracellular IP3 levels (Y. Yang et al., 2013). However
western blot analyses of LiCl-treated OSA cells showed a difficulty in producing interpretable LC3-I and II expression levels (data not shown). Moreover, LiCl interacts with numerous other pathways such as Wnt and GSK3-β pathways (Li et al., 2011). Therefore, a more specific approach was needed. L690,330, a lithium mimic was chosen due to its specificity as an IMPase inhibitor and was reported to be effective in restoring autophagy (Chang et al., 2011; Y. Yang et al., 2013).

Figure 8 shows four different concentrations of rapamycin used to treat both cell lines with FS and SF as positive and negative controls. Even at 1μM concentration, rapamycin successfully induced autophagy in both cell lines, indicated by the increased accumulation of LC3-II protein (Fig. 8). Interpretation of western blot of HCQ-treated cells was more challenging than rapamycin. Although all four treated groups had increased LC3-II levels (Fig. 9), indicative of autophagy activation, it is also important to observe LC3-I levels and compare with the SF group as well as remembering that HCQ could also be used to preserve LC3-II levels (Y. Yang et al., 2013). Since all HCQ-treated groups were cultured in SF condition, cells should undergo autophagy and LC3-I should be readily converted into LC3-II. Elevated LC3-I levels in a nutrient deficient/chemotherapy environment could indicate an inhibition of LC3-II conversion. In JL-31, all HCQ-treated groups had elevated LC3-I expression when compared to SF, particularly at 10μM and 20μM (Fig. 9). Results were not as clear in JL-75, where all except 10μM, had elevated LC3-II levels while 20μM and 50μM had slight increase in LC3-I levels when compared to SF. For L690, 330, treated groups of both cell lines experienced a dose-dependent increase in LC3-II levels (Fig. 10). However, only at 400μM were the treatment groups comparable to the rapamycin treated control. It was also interesting to see that even with elevated LC3-II levels, LC3-I levels seemed to be remain constant.

Autophagic responses and survival of PRKAR1A siRNA knockdown OSA cells after treatments: observation, justification and speculation

The involvement of PRKAR1A in cancer pathogenesis has been implicated in several cancer studies; however, the investigation of PRKAR1A has been challenging as the effects of PRKAR1A vary in
different cancers. In terms of OSA, it was shown that patients with low-PRKAR1A expressing OSA responded better to chemotherapy treatments than patients with high PRKAR1A expressing OSA in both human and canine (Larsen, 2011). In addition to this work, a study on tissues collected from CNC patients with inactivating-PRKAR1A mutations showed a significant build-up up of lipofuscin-like substrates (Mavrakis et al., 2007). These substrates are thought to be the residue of incomplete lysosomal degradation, indicative of autophagy deficiency (Mavrakis et al., 2007). In another study, it was also found that prkar1a−/− MEFs had reduced numbers of autophagosomes (Mavrakis et al., 2006). Within the same study, R1α KD HEK293 cells were found to have elevated phosphorylation of mTOR and mTOR kinase activity (Mavrakis et al., 2006).

Autophagy is often speculated to provide cells with cytoprotective effects as most dead cells have increased autophagosome formation (Moreau et al., 2010). While beneficial to normal cells, cancer cells could potentially take advantage of such effects and become resistant to chemotherapy. In Tasdemir et al., it was found that p53 intact cells could activate mTOR and reduce autophagy through AMP-dependent kinase while mutated p53 had increased cellular survival with enhanced autophagic response (Tasdemir et al., 2008).

We speculated that in canine OSA the longer MST of patients with low-PRKAR1A expressing OSA tumours was due to the reduced activation of mTOR and which led to a reduction in autophagy. The loss of autophagy rendered these OSA cells more susceptible to chemotherapy and consequently the patients had longer survival. To test this hypothesis, we had to establish three major elements: 1) PRKAR1A KD OSA cells had reduced autophagy when under chemotherapy treatment; 2) If the effect is mTOR dependent, mTOR independent rescue of autophagy should be able to reverse the effect; 3) chemotherapy-treated KD OSA cells had reduced survival and proliferation rate than control OSA cells. The first two parts could be measured through western blotting by determining relative LC3-I, LC3-II and p62 levels, as well as R1α levels for the confirmation of knockdown efficiency. Cells were either left untreated, or administered an IC50 of doxorubicin and/or L690, 330 for the stress and rescue. For the third
part, to investigate the differences in the ability of KD OSA cells to form colonies after treatments, a clonogenic survival assay was utilized. Clonogenic survival assays reflect clinical relevance as they determine the treatment effectiveness, particularly the ability of cancer cells to survive and proliferate.

Although LC3 bands in both cell lines exhibited different levels of expression, quantification of these bands gave us a very interesting result. In all treatment groups of JL-31 and JL-75, KD groups displayed lower autophagic flux than the NC1 groups (Fig. 11). The reduced autophagic flux in KD groups could indicate that the expression of PRKARIA is crucial for the conversion of LC3-I to LC3-II. As LC3-II is an important substrate of autophagy, the reduction in autophagic flux in KD could represent an incomplete autophagic response. However, the difference of autophagic flux between NC1 and KD did not translate into improved doxorubicin response, as shown in our clonogenic survival assays (Table 1–5). As the difference in autophagic flux was not substantial, it is speculated that the decrease in autophagic flux was not enough to provoke any meaningful clinical relevance.

In both JL-31 and JL-75 OSA cells, KD groups showed increased accumulation of LC3-I protein expressions under doxorubicin treatment when compared to NC1 groups (Fig. 11, 12). Failure of conversion from LC3-I to LC3II could be evidence of autophagic inhibition. Interestingly, similar expression levels where observed in L690 treated groups as well (Fig. 11, 12). As the expression of PRKARIA could interfere with major upstream regulators of mTOR, a low expression of PRKARIA through KD was thought to be able to reduce the activation of autophagy in a mTOR-dependent way (Mavrakis et al., 2007). Therefore, this effect could potentially be rescued by inducing autophagy independent of mTOR. Originally designed to be the rescue of autophagy through an mTOR independent pathway, as KD groups experienced an elevated LC3-I under L690 treatment as well, this suggested that this R1α expression level could interfere with the machinery of autophagy and not solely through mTOR as we initially hypothesized. Our results are consistent with previous findings of Mavrakis et al. where R1α proteins were found to be localized with LC3-positive autophagosomes (Mavrakis et al., 2006, 2007).
If R1α expression level is crucial to the formation of autophagosome, the cytoprotective effects of autophagy could potentially be eliminated through knockdown of PRKAR1A. Hence, KD OSA cells were hypothesized to be more susceptible to chemotherapy agents. However, clonogenic survival assays were not able to confirm this. Data analyses showed no statistically significant differences in either cell line when comparing the transfected groups and between each treatment (Fig. 14). This could be due to the limited sample size (n=3) and the wide margin of SEM between each treatment (Table 1). A statistical significance was particularly challenging to achieve when the SEM was almost 30% of the averaged mean in some samples. This massive SEM could be due to variables in culture and cell preparation conditions which interfered with the rate of proliferation of cell colonies.

**Limitations and alternatives**

A major limitation of this project is our approach in determining autophagic response. LC3 conversion is not inversely proportional; the decrease in LC3-I is smaller than the increase of LC3-II. Moreover, the expression level of LC3-II is often overestimated due to its higher immunoreactivity than LC3-I. Therefore, a comparison between LC3-I and LC3-I would not be appropriate. To address these problems, we could compare the amount of LC3-II among samples in terms of the total LC3 proteins produced. The band intensities of LC3-I and LC3-II were quantified and the autophagic flux was generated by LC3-II / (LC3-I + LC3-II). Detecting p62 expression levels during autophagy induction is an alternative to determine autophagic flux. p62 is accumulated during basal conditions and degraded along with the autophagic cargoes during autophagy. However, studies have shown that p62 expression level fluctuates independent of autophagy. Therefore, both LC3 conversion and p62 expression levels must be utilized in the same western blot to support our data.

Another limitation of this project is our method in quantifying changes of LC3 protein. In our experiments, all LC3 proteins were normalized to housekeeping protein, β-actin, before quantification. However, due to the abundance of β-actin in protein samples, subtle differences in protein loading are hard to detect and could contribute to misinterpretation of LC3 expressions. Moreover, in a study by
Reggiori et al., it was shown actin was consumed during certain types of autophagy (Reggiori et al., 2005). Although it is still uncertain how actin cytoskeleton contributes to autophagosome formation, the decrease in actin level could interfere with the LC3 quantification. An alternative approach is to visualise LC3 western blots with additional housekeeping proteins, such as tubulin and GAPDH. LC3 bands could be normalized to multiple housekeeping proteins to ensure linear loading of protein (D. Klionsky et al., 2016).

The third limitation of this project is the comparability to other studies. Currently, our LC3 primary antibody mainly detects LC3B –I and II proteins, a standard in detecting autophagy in western blot. However, LC3 proteins can be expressed in multiple isoforms, such as class A and C (Shpilka et al., 2011). As LC3 primary bodies from different manufactures could have various levels of affinity to LC3 isoforms, comparisons between different studies could be problematic. To establish a proper comparison, it is necessary to utilize the same primary antibody to eliminate potential inconsistencies (D. Klionsky et al., 2016).

Conclusion

siRNA-mediated PRKAR1A KD lead to increased LC3-I and decreased LC3-II expression, in agreement with a reduced autophagic response. However, clonogenic survival assay data showed a lack of evidence for any enhanced therapeutic effects of PRKAR1A KD. This result could be due to insufficient transfection efficiency, statistical variability in a limited sample size or it may be concluded that R1α KD alone is not enough to eliminate the cytoprotective/chemoresistant mechanisms of OSA cells. Given PRKAR1A’s role in autophagy supported by recent studies, it is highly plausible that this gene and its protein R1α will be a potential autophagy modulator to augment the effectiveness of current treatments in canine OSA. Nevertheless, more accurate and refined quantification methods of autophagy must first be developed to facilitate with the understanding of the relationship between R1α and cytoprotective effects of OSA cells.
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### APPENDIX I:
Fluorescence microscopy images of JL-31 and JL-75 OSA cells

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**Figure 17:** Fluorescence microscopy images of JL-31 and JL-75 OSA cells stained with GFP targeting autophagic substrates. Cells were seeded in 6-well plates with a cover glass in each well at 8 x 10^4 cells per mL for both JL-31 and 75. After 24 hours, cells allocated for transfection were transfected with either NC1 or PRKARIA siRNA. Other wells were left untreated. After 48 hours, positive control wells were treated with 1μM RAP while negative control wells were treated with 20μM HCQ. Cells transfected with siRNA were treated with doxorubicin IC_{50} of corresponding cell lines. Cells were treated for 24 hours. Cells were then stained with CYTO-ID® Green Detection Reagent and Hoechst 33342 Nuclear Stain. 400x images were captured by a fluorescence microscope using the FITC filter for GFP and DAPI for nuclear stain.
APPENDIX II:

Determining IC_{50} of Doxorubicin of JL-31 and JL-75 OSA cells for clonogenic survival assay

As cell viability assays measure metabolically active cells while clonogenic survival assays determine the proliferation ability of cells, the IC_{50} of doxorubicin determined by cell viability assay was not compatible with treatments in the clonogenic survival assay. Therefore, a separate IC_{50} was determined based on cell proliferation. Cells were seeded at 80,000 cells per mL in 12-well plates and incubated for 24 hours. Serial dilutions of doxorubicin (10nM to 60nM) were added into the wells and incubated for 24 hours. After treatment, the cells were washed with 1 X PBS, trypsinized and re-plated into 6-well plates in duplicates with standard culture media at 400 and 800 cells per mL for JL-31 and JL-75 respectively. After 10 – 14 days, media was removed from the wells. The wells were then washed with 1X PBS. The PBS was aspirated and 1.5mL of fixation solution was added into each well. After 2 hours, the fixation solution was removed and the plates were submerged in water. The remaining solution was carefully washed away and the plates were left to dry at room temperature. Colonies were counted manually using a stereomicroscope. A colony was defined to contain a minimum of 50 cells. Data from colonies formed in normal culture media was used to calculate the plating efficiency. Data from colonies formed under doxorubicin was normalized to plating efficiency to generate survival fractions. Survival fractions were then log-transformed and fitted on a sigmoidal dose-response curve. IC_{50} of each cell line was generated by GraphPad Prism. The IC_{50} of doxorubicin of JL-31 and JL-75 were determined to be 22.25nM and 28.68nM respectively.