NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

AN INNOVATIVE MAMMARY CANINE CANCER TREATMENT USING METAL COMPLEXES AND ITS MARKET TRANSFER STRATEGY

RITA DUARTE NUNES NEVES
Degree in Biochemistry

MASTER'S DEGREE IN BIOTECHNOLOGY
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ACKNOWLEDGEMENTS

I would like to thank everyone mentioned here for their support along this journey.

To begin with, I would like to thank my entire family for all their support, not just over the last year, but over these 17 years of education. For providing me with all the tools to achieve my goals, for always believing in my abilities and letting me pursue all my dreams. And to apologize for all the moments I missed from my role as granddaughter, daughter and sister.

I want to thank Professor Fernanda Llusá for this challenge she has given me and for the possibility of exploring a new area beyond the sciences. Thank you for all the teachings, patience and for all your availability. To Professor Alexandra Fernandes for giving me the opportunity to do an internship and develop my thesis in the HG&CT laboratory under her supervision.

Without a team by my side that could support me and teach me new things, this whole process would be more complicated, in this process the entire team from laboratories 315 and 319 of the Department of Life Sciences played an important role, and I am grateful to everyone they. I would especially like to thank Sandra Cordeiro who accompanied me throughout my study and always showed total availability to teach me and clarify all my doubts.

An important part of my thesis was the interviews/meetings with specialists in the areas of both research and veterinary medicine, I would like to thank all the specialists, both researchers and veterinarians, who took time out of their busy schedules in order to contribute positively to the development of my thesis.

To all my high school and university colleagues who always believed in my abilities and always motivated me to successfully complete this stage of my life.

Sport throughout this time has been my way of clearing my mind and keeping sane. Field hockey, which has been with me for 10 years, to all the great hockey family of Clube Futebol Benfica and especially to my dear team, of which I am very proud captain, my greatest thanks for all the understanding and help throughout this process.

I also want to thank my co-workers who supported me in this hard journey of writing a thesis and working at the same time. In particular, to my colleagues Ivo Cardoso and Catarina Alves, who, also being students-workers, showed all their support in the face of this challenge of mine.

Last but not least, I would like to thank my boyfriend, Guilherme, for all his patience and understanding over the years. A person who has been my strength and motivation many times, never letting me give up, believing in my abilities like no one else. Sorry for my crazy pace over the last year

and the many hours I couldn't be by your side when you needed it.

“Education is the most powerful weapon which you can use to change the world.”
-Nelson Mandela

ABSTRACT

Despite all the efforts that have been made to find better and more effective treatments to cure cancer, this disease remains one of the leading causes of death worldwide. The better understanding of cancer characteristics in recent years has also allowed the exploitation of new biological targets for treatment. The field of oncological research has been moving fast with new treatments and new molecules being discovered that can stop the growth of cancer cells avoiding damage to healthy tissues.

Previous data from the Human Genetics and Cancer Therapeutic Lab@FCT demonstrated that Cobalt and Zinc complexes with PhenDion (TS265 and TS262, respectively) have an excellent antiproliferative potential in a highly metastatic immortalized canine mammary tumor cell line, FR37-CMT. Moreover, both complexes were able to reduce tumor volume in human colorectal cancer xenografts with low accumulation in healthy tissues.

Based on these results, in this work, six metal complexes, all containing PhenDION as ligand, were tested against this metastatic canine tumor cell line, FR37-CMT.

Cell viability was assessed by the MTS Tetrazolium assay (MTS) and results indicated that JHOR9 and F (manganese) and VO(dipic)(DION) (vanadium) were the most highly cytotoxic with IC_{50} below 10 µM (JHOR9 (6.41 µM), F (8.20 µM) and VO(dipic)(DION) (0.28 µM)).The compounds seems to be more selective to cancer cells compared to normal cells. The loss of FR37-CMT cell viability could be attributed to the increase of reactive oxygen species and induction of autophagy.

Before proceeding to in vivo tests, the complex with greatest potential was selected based on a Value Creation Wheel (VCW) . Based on the filters used, results indicated that the vanadium complex was the most adequate to be further selected.

Therefore, and given that this research is at a very embryonic stage, deeper studies are still needed for a better definition of the most suited business model for the project. Regulations required in testing *in vivo* and research and development having in mind the cost benefit relationship to costumers are aspects that should be taken into consideration. The possibility of patenting and, later on, licensing the complex is a strategy that should not be discarded at this stage, as well as the possibility of exploring the technology as a spin off from Nova University.

Keywords: cancer; metal complexes; financial analysis; market research

RESUMO

Apesar de todos os esforços que têm sido feitos para encontrar tratamentos melhores e mais eficazes para curar o cancro, esta doença continua a ser uma das principais causas de morte em todo o mundo. O melhor conhecimento das características do cancro ao longo dos últimos anos, permitiu a exploração de novos alvos biológicos para o tratamento. O campo da pesquisa oncológica vem surgindo rapidamente com novos tratamentos e novas moléculas a ser descobertas que podem impedir o crescimento de células cancerígenas evitando danos aos tecidos saudáveis.

Dados anteriores do Human Genetics and Cancer Therapeutic Lab@FCT demonstraram que os complexos de Cobalto e Zinco com PhenDion (TS265 e TS262, respectivamente) têm um excelente potencial antiproliferativo em uma linha celular de tumor mamário canino imortalizada altamente metastática, FR37-CMT. Além disso, ambos os complexos foram capazes de reduzir o volume tumoral em xenoenxertos de cancro colorretal humano com baixo acúmulo em tecidos saudáveis. Neste trabalho, seis complexos metálicos (contendo DION como ligante) foram testados contra uma linhagem celular de tumor mamário canino imortalizada altamente metastática, FR37-CMT. Com base nesses resultados, neste trabalho, seis complexos metálicos, todos contendo DION como ligando, foram testados contra esta linhagem de células tumorais caninas metastáticas, FR37-CMT.

A viabilidade celular foi avaliada pelo ensaio MTS e os resultados indicaram que JHOR9 e F (manganês) e VO(dipic)(DION) (vanádio) foram os mais altamente citotóxicos com IC_{50} abaixo de 10 µM (JHOR9 (6,41 µM), F (8,20 µM) e VO(dipic)(DION) (0,28 µM)).Os compostos parecem ser mais seletivos para células cancerosas em comparação com células normais. A perda de viabilidade celular FR37-CMT pode ser atribuída ao aumento de espécies de oxigénio e indução de autofagia.

Antes de proceder aos testes in vivo, o complexo com maior potencial foi selecionado recorrendo à Value Creation Wheel (VCW). Com base nos filtros utilizados, os resultados indicaram que o complexo de vanádio foi o mais adequado para ser selecionado.

Assim, e dado que esta investigação se encontra numa fase muito embrionária, são ainda necessários estudos mais aprofundados para uma melhor definição do modelo de negócio mais adequado ao projeto. As regulamentações exigidas em testes in vivo e pesquisas e desenvolvimento tendo em vista a relação custo-benefício para os clientes são aspetos que devem ser levados em consideração. A possibilidade de patentear e, posteriormente, licenciar o complexo é uma estratégia que não deve ser descartada nesta fase, assim como explorar a tecnologia como um spin off da Universidade Nova.

Palavras chave: cancro; complexos metálicos; análise financeira; estudo de mercado.

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EQUATION INDEX

ABBREVIATIONS

A2780	Human ovarian carcinoma cell line
A2780R	Human ovarian carcinoma resistant to cisplatin cell line
A-498	Human kidney adenocarcinoma cell line
A549	Human lung carcinoma epithelial cell line
AC	Adenocarcinoma
ACN	Acridine
APAF-1	Apoptotic protease activating factor-1
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2 Associated X, Apoptosis Regulator
BCL-2	BCL-2 Apoptosis Regulator
BID	BH3 Interacting Domain Death Agonist
BMT	Benign mammary tumor
BPA	Boronated phenylalanine
BRCA1	Breast Cancer Type1 susceptibility protein
CAGR	Compound Annual Growth Rate
CCRF-CEM	Human acute T- lymphoblastic leukemia cell line
CCRF-SB	Human acute B- lymphoblastic leukemia cell line
CHANG	Human cervix carcinoma cell line
CIS	Cisplatin
CMT	Canine mammary tumors
COX-1	Cyclooxygenase- 1
COX-2	Cyclooxygenase- 2
CRL-7065	Human normal skin fibroblasts cell line
DA	Dodecylamine
DCF	2,7-dichloroflurescein
DDAVP	Desmopressin
DFS	Disease-free period
DGAV	Directorate-General for Food and Veterinary
DION	1,10-phenanthroline-5,6-dion
DIPIC	2,6- pyridinedicarboxylic acid
DMDP	4,4'-dimethyl-2,2'-dipyridyl
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DR5	TRAIL receptor 2
DU-145	Human prostate carcinoma cell line
EHR	Human epidermal receptor
EMT	Epithelial to mesenchymal transition
EVSA-T	Human breast carcinoma cell line
FAS	Fas Cell Surface Death Receptor
FCT-NOVA	NOVA School of Science and Technology
FR37-CMT	Canine mammary cancer cell line
H226	Human lung squamous carcinoma cell line
H2DCF-dA	Dichlorofluorescein-diacetate
HBC	Human breast cancer
HCT116	Human colorectal carcinoma cell line
HeLa	Human epitheloid cervix carcinoma
HEK-293	Normal human embryonic kidney cell line
Hep2	Human larynx carcinoma cell line
Hep-G2	Human hepatocellular carcinoma cell line
HG&CT	Human Genetics and Cancer Therapeutics Laboratory
HK-2	Normal Human renal cortex cell line
HMFN	Mefenamic acid
HNPR	Naproxen
HUVECS	Human umbilical vein endothelial cell line
IAC	Inflammatory adenocarcinoma
IGROV-1	Human ovarian cancer cell line

INFARMED	Autoridade Nacional do Medicamento e Produtos de Saúde
IPG	Isopentylglycine
ITQB	Instituto de Tecnologia Química e Biológica António Xavier
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimi-dazolyl-carbocyanine iodide
LPYA	Octadecylpyridine-2-yl methyl amine
M19-MEL	Human amelanotic melanoma cell line
MCF-7	Human breast cancer cell line
MCF10A	Human breast epithelial non-tumorigenic cell line
MDA-MB-231	Human breast cancer cell line
MMT	Malignant mammary tumor
MTS	[3-(4,5dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium]
NHL	Non-Hodgkin's lymphoma
NSAID	Non-steroidal anti-inflammatory drugs
OST	Overall survival time
PBS	Phosphate Buffered Saline
PC-3	Human prostate adenocarcinoma cell line
PG	Prostaglandin
PI	Propidium Iodide
PNT	5-phenyltetrazole
PS	Phosphatidylserine
PTA=O	1,3,5-triaza-7-phosphaadamantane-7-oxide
PWAP	Peptides with antitumor proprieties
RAW 264.7	Mouse macrophage cell line
R&D	Research and development
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
RTKI	Receptor tyrosine kinase inhibitor
SK-MES-1	Human squamous cell carcinoma cell line
STS	Soft tissue sarcomas
T25	25 cm^2 vented cell culture flask
T98G	Human glioblastoma cell line
TBID	Truncated BID
Trail	TNF-related apoptosis-inducing ligand
UNL	NOVA University of Lisbon
USA	United States of America
VCW	Value Creation Wheel
WIDr	Human colorectal carcinoma cell line

Symbol List

[Compound]	Compound concentration
ΔΨM	Mitochondrial membrane potential
IC_{50}	Half Maximal Inhibitory Concentration

CHAPTER 1: INTRODUCTION

1.1. Cancer: incidence and models for the study of human cancer

Cancer is a group of diseases, with more one hundred different cancer types, characterized by the emergence of abnormal cells that divide and expand uncontrollably in any part of the body, invade nearby tissues, and migrate to other organs, causing harm to healthy tissues in a process called metastization (Cekanova and Rathore, 2014; Rojas, 2017; Seyfried et al., 2010; Welch and Hurst, 2019).

The ordered renewal of cells in a healthy body enables our body's tissues to heal and grow in harmony. Mutations in crucial genes associated with cell cycle progression and/or DNA repair induces permanent damage in the genetic material, which drives cancer development. The next stage results in the unchecked growth of abnormal cells, not recognized as abnormal by the immune system, and disease progression. (Seyfried et al., 2010). Throughout its multistep development, cancer has been characterized by several characteristics, that will highlighted in more detail in section 1.2, and include maintaining proliferative signalling, inhibiting growth suppressors, enabling replicative immortality, resisting cell death, tumor-promoting inflammation, inducing angiogenesis, activating invasion and metastasis, genome instability and mutations, dodging immune destruction, and deregulating cellular energetics (Cekanova and Rathore, 2014).

Worldwide, the incidence and death of cancer are rising at a faster speed. In 2020, about 10 million people died with cancer deaths and 19.3million new cases appeared worldwide (Sung, 2021).

Diet, smoking, virus infections, excessive radiation exposure, exposure to specific chemicals and hormones, and genetic influences are just a few of the variables that might cause cancer to develop. Up until the disease is recognized, these causes may function singly or in combination over a long period of time (Akram et al., 2017). For example, in breast cancer, a diet high in fat and poor in fiber, as well as alcohol consumption, are frequently linked to preventable risk factors (apart from age-related factors). Because adipose tissue encourages the synthesis of estrogen and other hormones that either stimulate or inhibit cell proliferation, being overweight is also linked to an increased incidence of breast cancer (Akram et al., 2017). The understanding of factors that may contribute to cancer is important for its prevention. To study cancer and to develop new treatment regimens good models are necessary.

In carcer research, animal models and human/animal primary and/or immortalized cancer cell lines have been used to fully assess cancer complexity. . Highly controlled circumstances, homogeneity, the identification of molecular pathways, and reproducibility are benefits of using *in vitro* cancer models. However, the selection of phenotypic and genotypic cellular populations during adaptation to *in vitro* conditions, accumulation of mutations in cells over time, and the loss of the tumor microenvironment in

vivo complexity are some of the main drawbacks of using this in vitro cancer models. Despite the mentioned drawbacks, this has been and will continue to be the primary system model of choice for cancer studies (Cekanova and Rathore, 2014; Selo et al., 2021).

Animal models have been used over the years as a key component of numerous research on cancer, identification of targets, and evaluation of novel therapeutic agents and treatments (Lannagan et al., 2021), however, the 3Rs policy (Replacement, Reduction and Refinement) poses some limitations to the use of these models Yadav and Singh, 2021). Moreover, the development of tumors in mice is not a natural phenomenon as in man, dog, and cat counterparts (Pang and Argyle, 2009). For example, in xenograft cancer models, immunocompromised mice are used to implant human or animal cancer cells beneath the skin or directly into the organ to give rise to a tumor meaning that their immune systems do not behave as in a natural cancer model (Cekanova and Rathore, 2014). So careful must be used for these interspecies (e.g. mice) translational studies in what concerns drug development for human application. In companion animals such as dogs, cancers occur in animals with an intact immune system. Tumors are heterogeneous, develop recurrent, drug-resistant disease, and metastasize to distant sites. These tumors capture the essence of human cancer better than any other model system (Cekanova and Rathore, 2014).

In this regard, choosing an approach based on a natural animal model will also allow us to approach the veterinary industry, which is also a growing industry (Hernandez et al., 2022). Companion animals fill the gap between *in vitro* and *in vivo* studies in the pet population by having many desired traits, and these traits have proven crucial in understanding many intricate molecular components of human cancer (Garden, 2018).

Due to similarities in clinical behavior, biological traits, epidemiology, histological morphology, molecular targets, hormonal etiology and genetics, prognostic factors, mode of metastatic progression, and response to conventional treatments, it is already well established that the bitch is an excellent model of study for breast cancer in women. The fact that dogs are exposed to carcinogens in the shared environment (an important factor in the development of cancer) with humans, as well as the fact that the genomic map of dogs has been found to approach surprising human, support the idea that female dog mammary cancer is a good model for spontaneous breast cancer in women (Gray et al., 2020; Timmermans-Sprang et al, 2017).

Mammary neoplasms are the most prevalent tumor in female canines and breast cancer is the second most frequently diagnosed disease in women. The hormone reliance, pattern of metastatic spread, age, and contribution of environmental factors to disease start are just a few of the similarities between human and canine mammary cancers (Cekanova and Rathore, 2014). In section 1.3 canine mammary tumors will be highlighted in more detail.

1.2. Molecular Biology of Cancer

As previously explained, cancer begins as a disease of the genome at the cellular level. The development of a carcinogenic tissue is a complex process that is related to the sequential accumulation of genetic mutations in the cells that constitute the tissues, leading to the deregulation of gene activities, essential for healthy tissue development, involved in the control of cell growth, regulation of programmed cell death. and maintenance of genetic stability (Pecorino, 2021).

1.2.1. The hallmarks of cancers

Hanahan and Weinberg identified six hallmarks that contribute to the emergence of cancer in the early 2000s, including signs of autonomous growth, evasion of inhibitory growth signals, evasion of apoptosis cell death, unlimited potential for replication, angiogenesis (the growth of new blood vessels), invasion, and metastasis. Years later, to these six hallmarks were added, two emerging hallmarks. More research in this field to understand their relationship to the previously required pre-defined hallmarks and their role in the genesis of cancer is needed, these emerging hallmarks, are reprogramming energy from the metabolism and preventing immune destruction (Figure1) (Hanahan and Weinberg, 2011; Pecorino, 2021).

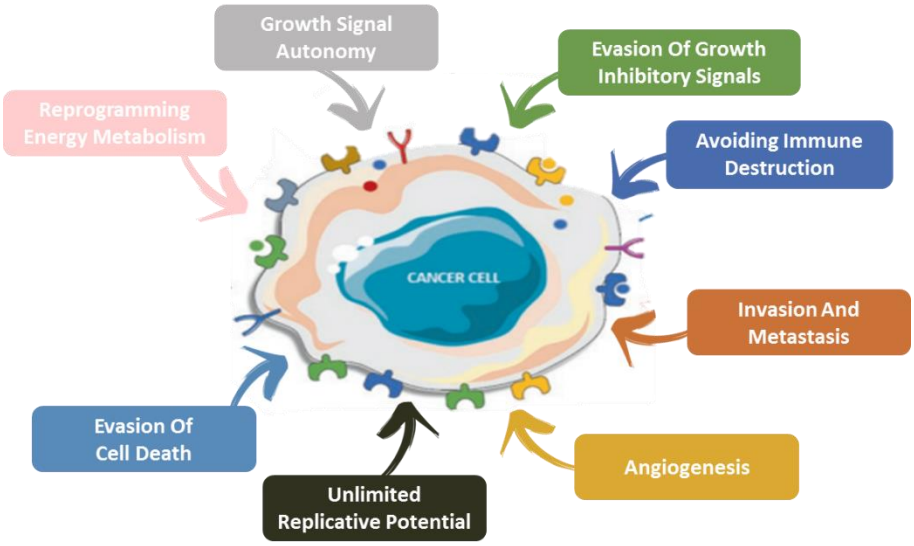

Figure 1: Hallmarks and emerging hallmarks of cancer (Adapted from: (Pecorino, 2021))

These hallmarks of cancer cells result from mutational changes that give them distinctive properties that enable their continuous growth and development (Pecorino, 2021).

The search for new cancer characteristics was not stationary, and more recently, in 2021, 4 more additional hallmarks were suggested as likely to be added to the already known ones, these being: dedifferentiation/ transdifferentiation, epigenetic desregulation, altered microbiomes and altered nerve signaling (Pecorino, 2021; Senga and Grose, 2021).

Most antitumor drugs used in therapy affect the process of cell division, their main effect is exerted on cell division, also affecting normal tissues with rapid division, producing adverse effects. Lack of selectivity against tumor cells versus normal cells is the main problem with conventional medicines and their underlying therapies. An analysis and a good knowledge of these hallmarks are important to find new possible targets for cancer therapies and help to design new possible oncological drugs (Pecorino, 2021).

1.2.2. Programmed cell death mechanisms

One of the hallmarks of carcinogenesis is the resistance to death by apoptosis in cells that have escaped the control of normal growth and differentiation exerted by soluble factors or by cell-cell contacts or cell-extracellular matrix up to that induced by DNA damage, hypoxia, or reactive oxygen species (ROS), has been explored over the years with a view to developing new therapies to fight cancer, as well as exploring another program of death mechanism, autophagy. In the following subsections we will address these cell death mechanisms (Grivicich et al., 2007; Pecorino, 2021).

1.2.2.1. Apoptosis

Apoptosis, Type I programmed cell death, is a highly controlled physiological mechanism of cell death not only affects developmental morphogenesis but also regulates cell proliferation and gets rid of damaged cells. This is a mechanism that is crucial in the suppression of malignancies because apoptosis destroys cells with substantial genetic damage, cells that might later grow into cancer (Pecorino, 2021; Thorburn, 2008).

Cell shrinkage, membrane blebbing and budding, chromatin condensation, and fine fragmentation are all signs of apoptosis and all contribute to the net destruction of the cell. Contrast this with the "messy" process of necrosis, in which cells enlarge, cell walls become permeable, and cells exude their contents into the surrounding tissue, triggering inflammation. External factors like infections or injuries can lead to necrosis (Pecorino, 2021).

Cells can undergo apoptosis in response to both external signals and internal physical or chemical stimuli, such as oxidative stress or DNA damage. There are two different pathways possible in apoptosis, extrinsic and intrinsic molecular pathways, which are different but not mutually exclusive. Caspases are essential components of both routes and are present in both. These proteases are produced by procaspases, dormant proteins that must be cleaved into aspartate residues in order to become active. The intrinsic pathway is started by cellular stressors that cause the release of cytochrome-c from mitochondria, while the extrinsic mechanism involves ligand interaction to a death receptor (Figure 2) (Millimouno et al. 2014; Pecorino, 2021).

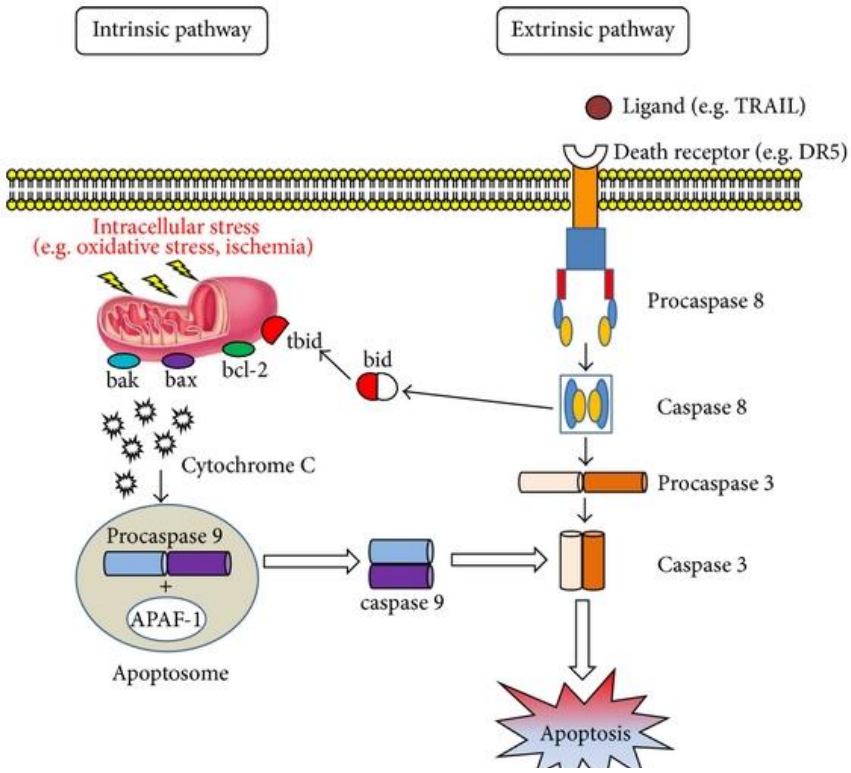

Figure 2: Extrinsic and intrinsic pathways of apoptosis cell death. The schematic diagram describes the signalling molecules involved in the extrinsic (death receptor) and intrinsic (mitochondrial) apoptosis pathways. (Source: Loreto et al., 2014)

A transmembrane cell death receptor recognizes a death factor, such as FAS ligand or tumor necrosis death factor, in the extrinsic pathway of apoptosis. To transmit the signal into the cell after this interaction, the receptors go through a conformational shift and form homotrimers. Procaspase-8 molecules are recruited, and those that are near to one another become activated by self-cleavage. These activated caspases encourage the release of active enzymes into the cytoplasm, allowing the cleavage and activation of caspase effectors including caspase-3, and starting the sequence of events leading to apoptotic cell death (Millimouno et al. 2014; Pecorino, 2021, Ranjan et al., 2012).

The intrinsic process of apoptosis can be activated without the aid of an outside stimulus. Interior stimuli activate this apoptotic pathway through BCL-2 family members that operate on the outer membrane, as was already explained. The proteins in this family may promote or inhibit apoptosis in opposing ways. Healthy cells have a protein called BCL-2 on their mitochondrial membranes, which normally inhibits apoptosis. When cell is damaged, it stimulates a protein, BAX, to move onto outer mitochondrial membrane and inhibits BCL-2. The mitochondria are the primary target of the signals that are transduced in response to these stimuli. By integrating cell death stimuli, this organelle causes mitochondrial permeabilization and the subsequent release of pro-apoptotic chemicals. This entire process is started by the permeabilization, promoted by BAX, of the mitochondrial membrane, there is a collapse of the potential of the inner mitochondrial membrane ($\Delta\psi$) and the release of pro-apoptotic substances that have been accumulated and a conformational change occurs. These stimuli cause

increase the mitochondrial secretion of cytochrome-c into the cell cytoplasm, when this binds to and activates procaspase-9 and apoptotic protease activating factor 1. This pathway is characterized by the loss of cellular homeostasis, interrupting ATP synthesis and increasing the production of ROS, and induction of caspases. Then, the apoptosome activates caspase-3, which leads to the cleavage of proteins. Through the cleavage of Bid by caspase-8, the intrinsic and extrinsic apoptosis pathways interact. (Galluzzi et al. 2012; Grivicich et al., 2007; Pecorino, 2021).

All of these changes lead to the cellular fragmentation that preserves organelle integrity by retaining the cytoplasm in subcellular apoptotic bodies. The apoptotic bodies are then phagocytosed by macrophages and broken down on phagolysosomes after that (Grivicich et al., 2007; Pecorino, 2021)

1.2.2.2. Autophagy

The metabolic pathway known as autophagy, also known as Type II programmed cell death, is a dynamic, multistep process that involves the degradation of protein aggregates, lipids, ribosomes, and damaged or unneeded organelles, is characterized by dynamic membrane rearrangements and crucial for maintaining cellular homeostasis and has a variety of physiological and pathological functions. Due to its potential as a therapeutic target, particularly for cancer, where it operates in an ambiguous manner and can either repress or stimulate the tumor depending on the situation, it has been the subject of numerous investigations (Do Nascimento Silva and Martins, 2020; Thornburn, 2008).

During autophagy, senescent organelles and cellular proteins are sequestered in autophagic vacuoles, which subsequently bind to lysosomes, where they are ultimately degraded, and whose degradation products are reused by anabolism. Defects in this process have been commonly associated with various diseases such as cancer (Do Nascimento Silva and Martins, 2020; Galluzzi et al., 2017; Saha et al., 2018).

There are three main types of autophagy: microautophagy, chaperone-mediated autophagy and macroautophagy (Do Nascimento Silva and Martins, 2020).

The entire process of autophagy by activating signaling molecules, protein kinases (including the mTOR 1 complex) relay information about the metabolic condition of the cell, become activated and signal to the autophagic machinery. After induction of autophagy, the formation of a double membrane structure called a phagophore occurs in the cytoplasm (Figure 3). The phagophore elongates enveloping organelles and macromolecules, becoming a double membrane vesicle, which after maturation is called an autophagosome (Do Nascimento Silva and Martins, 2020; Yu et al., 2018). A membrane precursor, such as an autophagopore formed from the ER's lipid bilayer, the trans-Golgi network, the mitochondrial outer membrane, or the plasma membrane, is necessary for the creation of autophagosomes. The membrane precursor subsequently expands to engulf the intracellular components. This, after fusing with the lysosome, becomes an autolysosome capable of degrading the vesicular contents due to the action of lysosomal hydrolases (Do Nascimento Silva and Martins, 2020). The by-products are released

back into the cytosol where they can be utilized again for metabolism and the synthesis of new macromolecules (Glick et al. 2010).

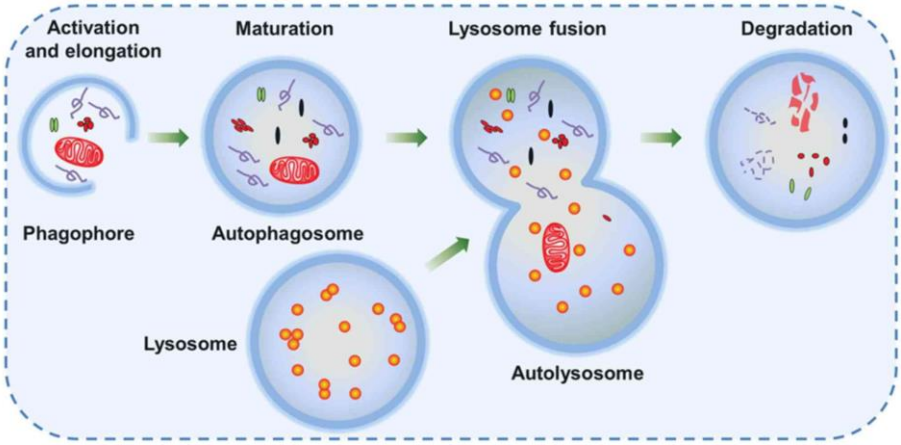

Figure 3: Autophagy process (Source: (Li et al., 2019))

1.3. Canine mammary tumors: an overview

Domestic animals are developing neoplasms at an increasing rate, either as a result of longer lifespans or the development of diagnostic and therapeutic techniques (LeBlanc,2020).

About 25% of dogs will experience neoplasia at some point in their lives, and over 50% of dogs over the age of 10 will experience malignant neoplasms, or cancer. CMT are the most prevalent neoplasm in female dogs, accounting for 52% of all neoplasms, and the second most common tumor in dogs, only after skin cancer (Candellone et al., 2021). Due to the structure of the canine lymphatic system, the local lymph nodes and lungs are most likely to be afflicted by metastasis since they are approximately 50% malignant and have a higher likelihood of spreading to other parts of the body. Less frequently than the liver, kidney, bone, skin, brain, and adrenal gland, cancer metastasizes into these organs (Clemente et al,2010).

Only 1% of male dogs have this condition, making it an unusual occurrence in male canines. CMTs are prevalent in both sterilized and post-first heat cycle sterilized dogs (Kaur et al., 2022). Older or middle-aged female canines are more likely to get the tumor (Zatlouka et al, 2005). Approximately 70% of canine females get malignant tumors (Valdivia et al, 2021).

There is still much to learn about the precise reasons why dogs acquire mammary tumors. It's common knowledge that exposure to certain hormones, like progesterone, raises the possibility of developing cancer. Because progesterone promotes growth factors that lead to mammary cell duplication (Rao et al, 2009).

There is little data on the lifetime morbidity and mortality of female client-owned dogs related to female reproductive pathology. Mammary cancers are said to be prevented by spaying before the first

or second oestrus in older research. But over the past ten years, it has been noted that the statistical reliability of these has been low, and numerous epidemiological studies have since demonstrated that early spaying of female dogs, depending on the breed, is significantly linked to an increased risk of osteoarthritis, cruciate ligament rupture, immune-mediated diseases, epilepsy disorders, and/or some cancers (Beaudu-Lange et al, 2021)

It seems that some breeds are more frequently impacted. There is still much to learn in veterinary medicine regarding the genes and mutations that might cause canine mammary cancers (Kaszak et al, 2018). Research in humans has demonstrated a clear link between the BRCA gene and the emergence of breast cancer (Eccles, 2015).

Dogs have a roughly three times higher incidence of cancers than humans do. Many environmental, ecological, clinical, genetic, and pathological characteristics, including a striking histological and molecular heterogeneity, are shared by canine mammary and human breast cancer. CMT has been hailed by many authors as an effective spontaneous model for the study of HBC (Valdivia et al, 2021)

1.3.1. Characterization

CMTs are the neoplasms with the highest degree of morphological variety, resulting from a wide range of cell types that are frequently present in the same tumor (Gray et al., 2020) The three types of them are carcinomas, sarcomas, and carcinosarcomas. Carcinomas are malignant epithelial or myoepithelial CMTs that develop from epithelial cells, mammary gland tubules, or other cells found in the mammary chain. These tumors are divided into four categories: in situ (non-infiltrating), complex (involving myoepithelial cells), simple, and mixed (with mesenchymal cells involved) (Canadas et al, 2019; Raposo et al, 2017). Malignant CMTs with mesenchymal origin known as sarcomas include fibrosarcoma, osteosarcoma, and other breast cancers. The final one, known as carcinosarcoma, is a rare variety of breast mixed tumor in which the tumor's mesenchymal and epithelial components are both cancerous (Raposo et al, 2017).

The most frequent of these malignant CMTs are epithelial carcinomas (Raposo et al, 2017).

The dog's mammary cancer, as well as the woman's, affects in more advanced stages of life and the diet practiced, as well as the dogs' constitution, demonstrate to have an influence on the development of the disease. CMT can also show significant histological variation both within a single individual tumor and between various tumors in the same dog. This can happen in more than one gland (often females present five pairs). There is a substantial likelihood that canine mammary carcinomas will spread to nearby lymph nodes, metastasizing, which is the biggest cause of death associated with this type of cancer (Gray et al., 2020).

The mammary ridge in female dogs is usually built up of five pairs of mammary glands. The mammary chain is a collection of five glands that run either down the left or right side of the body. The next sets of pairs of glands are the cranial thoracic, caudal thoracic, cranial abdominal, caudal abdominal, and inguinal (Borecka et al., 2020).

The majority of CMTs, 70%, are found in older, intact female canines, and they most frequently involve the caudal abdominal and inguinal mammary glands, as well as numerous tumors in one or both mammary chains. Patients who have had prior CMTs are more likely to acquire new mammary tumors, which are frequently situated on the same side as the prior one (Raposo et al, 2017). As in HBC, differences in the number of genomic copies are the genetic alteration more common in CMTs (Raposo et al, 2017).

CMT development is impacted by steroid hormones. Additionally, it appears that progesterone and estrogens are necessary for CMTs to develop malignant behavior (Raposo et al, 2017).
Mammary neoplasias have an unknown specific cause, however it is thought that genetic, environmental, dietary, and hormonal variables have a role in their development (Andrade et al, 2010; Diamantini-Kandarakis et al, 2009).

1.3.1.1. Hormonal Factors

It is well recognized that ovarian hormones, particularly estrogens and progesterone, are important in the development of CMT. As a result, the age at which a female dog has her ovariectomy affects the likelihood that she will acquire malignant tumors. Felines that are neutered after the second estrous cycle had a 26% risk, compared to 8% for those that are neutered after the first estrous cycle in chihuahuas (Benavente et al, 2016, Howe, 2015).

Steroid hormone levels were found to be higher in malignant mammary tumor tissue homogenates than in benign ones, suggesting that these hormones may operate as local growth factors in the malignant forms, promoting their proliferation (Benavente et al, 2016, Queiroga et al, 2005). Other hormones and growth factors may also have an impact on the growth of breast tumors in addition to steroid hormones (Benavente et al, 2016).

1.3.1.2. Genetic Factors

Mammary tumor formation is influenced by genetic changes, according to several studies, and some breeds may be more predisposed to the cancer than others (Benavente et al, 2016, Dobson, 2013). Compared to other breeds, tiny breeds appear to have a decreased incidence of malignant tumors (Benavente et al, 2016).

Malignant and benign CMT have been found to have HER-2 mutations and overexpression; research suggests that this mutation increases angiogenesis in mammary carcinomas (Benavente et

al, 2016; Kaszak et al, 2018; Klopfleisch et al, 2011).

P53 is another gene implicated in the development of breast tumors. This tumor suppressor gene produces both benign and malignant p53 mutations, which affect the protein that usually controls cell cycle and programmed cell death (Benavente et al, 2016; Klopfleisch et al, 2011).

Mutations in the breast cancer 1 tumor suppressor gene have been found in CMT. As a nuclear protein, BRCA1 may exhibit an aberrant cytoplasmatic distribution if its function is lost. BRCA1 expression is related with cancerous features in dogs when it is absent (Benavente et al, 2016; Nieto et al, 2003).

Nuclear DNA abnormalities have been identified in CMT, more frequently in malignant than benign varieties. In fact, dogs with identified malignant mammary tumors have lower survival chances when their DNA is aneuploid (Benavente et al, 2016).

1.3.1.3. Nutritional Factors

Since adipose tissue can produce several steroid hormones, nutritional considerations are of some importance. Due to its aromatase activity, which changes androgens into estrogens, adipose tissue is a significant source of estrogens. Increased breast adipose tissue increases to the gland's exposure to estrogens, which is known to encourage the development of mammary tumors. Studies showed a strong link between obesity and an increased risk of breast cancers (Benavente et al, 2016; Wang et al, 2015).

1.3.1.4. Environmental factors

The presence of environmental pollutants may affect the genesis of mammary tumors and the development of breast cancer in dogs (Andrade et al, 2010; Vascellari et al, 2016).

1.3.2. Treatments

The most popular therapeutic approach is surgical excision, which is the preferred course of treatment but in many cases, the procedure alone is ineffective due to existing micro-metastases. Additionally, potential alternative treatment approaches are not possible due to the lack of efficient chemotherapy and radiotherapy protocols. Whereby, There has been a noticeable and expanding interest in this topic in fetuses, leading to the discovery of other therapeutic strategies targeted at more precise targets (Timmermans-Sprang et al, 2017)..

1.3.2.1. Conventional therapy

Most procedures utilized for canine patients are based on treatments provided for human patients because CMTs and HBC are extremely similar. Early detection and swift treatment are necessary to stop local or remote transmission (Novosad, 2003).

Except for circumstances when surgery is impossible, such as inflammatory carcinoma and distant metastases, mastectomy surgery is the preferred treatment for CMT (Valdivia et al, 2021, Benavente et al, 2016). Adjuvant therapies, such as chemotherapy, are used in cases of patients with the most advanced/aggressive stages of the disease. Adjuvant therapy haven't yet been shown to clearly assist dogs with CMT, though. Dogs with mammary neoplasms do not frequently receive precise therapy (Valdivia et al, 2021).

Over 40% of patients with CMT die within a year of their diagnosis, which is a rather high mortality rate (Valdivia et al, 2021). Current treatment protocols are ineffective, as evidenced by the continually high mortality rate of female dogs following surgical removal of malignant mammary tumors (Stratmann, 2008).

1.3.2.1.1. Surgery

Surgery continues to be the major method of controlling CMTs. The dog has undergone a variety of surgical techniques, from local excision to radical excision. It is anticipated that all afflicted tissue will be removed with clean margins, and in certain situations, future tumor growth in the residual glands will be stopped (Clemente et al, 2010; Raposo et al, 2017; Novosad, 2003).

There seems to be no difference between a standard mastectomy and a radical procedure in terms of recurrence rates or survival durations (Novosad, 2003).

Other therapies can be administered following surgery to reduce the possibility of developing future metastases and recurrences (Raposo et al, 2017).

1.3.2.1.2. Chemotherapy

There seems to be no difference between a standard mastectomy and a radical procedure in terms of recurrence rates or survival durations (Novosad, 2003).

Is occasionally advised for mares with invasive mammary cancers and a high risk of metastasis or recurrence (Benavente et al, 2016).

However, there is no one chemotherapy treatment plan that demonstrates the effectiveness of chemotherapy in the management of canine mammary cancers. This is typically the therapy of choice

in people. One of the most effective treatments for individuals with chronic conditions is doxorubicin. *In vitro* models have demonstrated some activity of this medication against CMT cell lines. Both this medication and docetaxel have been tried in the treatment of MMT, but neither had any success (Benavente et al, 2016; Novosad, 2003).

The risk of unfavorable tissue effects such the production of reactive oxygen species leading to oxidative stress and cellular alterations from DOX remains a top concern (Abdelmegeed and Mohammed, 2018).

Following mastectomy, an *in vivo* study of a chemotherapy program using cyclophosphamide and 5-fluorouracil showed promising outcomes in terms of the time that the treated dogs remained tumor-free and their survival rates (Benavente et al, 2016).

1.3.2.1.3. Radiotherapy

Radiotherapy may be utilized in a palliative setting for nonresectable or inflammatory mammary carcinomas or to help manage local illness for tumors that have been partially removed (Novosad, 2003).

This treatment modality is not available in the Portuguese territory. When dogs need to practice this type of treatment, they must be transferred to clinics outside the country, to countries like Spain and France, which entails high costs, which many people cannot afford (Appendix 3).

1.3.2.2. Precision therapy

Research advancements in cancer have made it possible to identify previously unidentified chemicals implicated in the carcinogenic process and design treatments intended to prevent these compounds (Benavente et al, 2016).

1.3.2.2.1. Hormonal therapy

Because of this tumor's hormonal reliance, hormone-based therapy may be an option to examine (Benavente et al, 2016). In individuals with estrogen receptor-positive breast cancer, hormonal therapy is a generally approved treatment option that prolongs patient survival. (Novosad, 2003; Benavente et al., 2016)

Despite the documented dependence of CMT development on estrogens and progesterone, the use of antiestrogens such as tamoxifen (selective estrogen receptor modulator) showed significant activity in humans but inconsistent outcomes in tests with dogs. Such therapies may have negative effects on the reproductive organs, such as vulvar enlargement, vaginal discharge, pyometra in intact females, and stump pyometra in females who have had spaying (Raposo et al, 2017; Benavente et al,

2016; Novosad, 2003).

The use of PR antagonists found in MMT as adjuvant therapy appears to be an option. Aglepristone has demonstrated efficacy in preventing canine mammary cancer cells that are PR positive from proliferating (Benavente et al, 2016).

In test therapy utilizing gonadotropin-releasing hormone agonist, the size of the tumors was reduced and the survival time in cases with hormone-dependent mammary carcinomas was improved (Benavente et al, 2016).

1.3.2.2.2. Non-steroidal anti-inflammatory drugs

One of the two isoforms of prostaglandin synthases is cyclooxygenase-2 (COX-2). Unlike COX-1, which is a constitutive enzyme that ensures the synthesis of prostaglandins required for physiological functions in the majority of tissues, COX-2 is an enzyme induced by growth factors, tumor promoters, and inflammatory processes. It has been proven that this enzyme contributes to the growth and spread of tumors. COX-2 is expressed in a wide variety of canine tumors, including those of the mammary gland. The PGs made by COX-2 boost the tumor's susceptibility to apoptosis, promote angiogenesis, spur tumor cell growth, and inhibit the immune system (Benavente et al, 2016).

Due to COX-2's role in the tumor, selective COX-2 inhibitors have been suggested as a possible treatment. *In vitro* tests have shown that the anti-inflammatory drugs piroxicam and meloxicam can stop the growth of breast cancer cell lines. There have already been *in vivo* studies with piroxicam that showed an improvement in the patient's health and superior outcomes to those seen with conventional treatment (Benavente et al, 2016).

1.3.2.2.3. Peptides with antitumor proprieties

As a selective agonist for the vasopressin 2 membrane receptor, desmopressin, also known as 1-deamino-8-d-arginine vasopressin, is a synthetic peptide analog of the antidiuretic hormone vasopressin. DDAVP is already used to manage diabetic insipidus and possesses hemostatic and antidiuretic properties. Additionally, this peptide has shown antiproliferative activities in human breast cancer cell lines and antimetastatic effects in a mouse model (Benavente et al, 2016).

When given to dogs with breast cancer following mastectomy, DDAVP had a positive impact on their overall survival and time spent free of disease, with no negative side effects (Raposo et al, 2017; Benavente et al, 2016)

1.3.2.2.4. Receptor tyrosine kinase inhibitor

Tyrosine kinase receptors, are overexpressed or constitutively activated in canine tumors,

catalyze a series of phosphorylation of target proteins that play a significant role in cell proliferation, metabolism, motility, survival, and apoptosis, as well as endothelial cell activation, leading to neovascularization (Raposo et al., 2017; Valdivia et al., 2021).

Tyrosine kinase inhibitors cause the dysregulation of cellular proliferation and differentiation by competitively inhibiting ATP binding. The majority of TKIs are administered orally, which has significant positive effects on animal welfare by reducing stressful conditions and making administration by the owner simpler (Valdivia et al., 2021).

1.3.2.2.5. Anti-Her-2

HER-1, HER-2, HER-3, and HER-4 are the four tyrosine kinase receptors found on human epidermal receptors. These receptors drive a variety of signaling pathways, which control tumor cell metastasis as well as cell survival and proliferation (Valdivia et al., 2021).

In certain cases of breast cancers, the HER-2 gene has been amplified or the HER-2 protein has been overexpressed (Valdivia et al., 2021).

While several anti-HER-2 drug classes have been created for HBC, only gefitinib has been shown effective in CMTs. A canine mammary cancer cell line demonstrated anti-proliferative effects in *in vitro* tests. The use of HER-2 inhibitors in CMT has not been the subject of any published clinical studies (Valdivia et al., 2021).

1.3.2.2.6. Antitumor Suppressor Gene p53

The P53 protein prevents the growth of new cells by starting cell cycle arrest and death. Its tumor-suppressor activity is frequently disrupted by mutations, which also result in genomic instability. Numerous malignancies in dogs have been linked to p53 gene mutations (Valdivia et al., 2021).

P53 levels have been shown to be higher in malignant tumors than benign tumors, and they have also been found to be higher in higher-grade tumors with higher rates of proliferation. Increases in p53 expression are significantly correlated with mutations associated with shorter OS (Valdivia et al., 2021)..

With KPT-185 and KPT-355, designed compounds that block exportin-1 on canine mammary carcinoma cells, *in vitro* induced cell cycle arrest, death, and decreased proliferation, there is the only one research on p53 treatment in canine mammary cancer cells (Valdivia et al., 2021).

1.4. Metal complexes in medicine

Most compounds investigated in modern medicine are based on organic molecules. Despite all the advancements in several fields, such as oncology, the mortality rates for the same have not much improved, necessitating new treatment approaches. In recent decades, more attention has been paid to metal complexes and their potential. These compounds offer different mechanisms of action compared to organic molecules due to their unique properties, making them new drug candidates (Karges, 2020).

Metal complexes offer distinctive properties, such as high structural diversity, redox properties, catalytic properties and tendency to ligand exchange, in comparison to organic compounds that enable unique modes of action. The inability to distinguish between therapeutic and hazardous doses, however, continues to be a significant problem for the majority of the medicinal scientists. However, it is impossible to generalize about a complex's lethality because it depends on a variety of variables, including the metal used, the oxidation state, the coordination geometry, and the kind of coordinated ligands (Karges, 2020)

This type of compounds seems to be of little practice in the field of veterinary oncology, there are still few studies in this regard and cisplatin is regularly used in the treatment of cancer, despite all its disadvantages (Appendix 3).

More information on the state of art in relation to metal complexes will be explored in Chapter 2.

1.5. Market analysis

The veterinary market can be said to be expanding, with the field of veterinary oncology also growing, with an expected increase in market value of almost 400% in 10 years (Dash and Suman, 2021). This growth is largely explained by the importance that four-legged companions have come to represent in families, being seen in them as another member of the family, who must have access to the same care as humans (Appendix 3).

Despite the investments made over the years in the field of oncology, there have been no significant effects in reducing deaths. More relatively, focusing on canine breast cancer, surgery remains the treatment of choice, often not being enough, but other adjuvant treatments have not been shown to have efficient protocols (Stratmann, 2008).

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These data reveal the need to investigate new treatments in this area, thus revealing a "fertile soil" to invest with metal complexes.

1.6. Translational medicine- From dogs to humans

Numerous studies and proposals for animal models for studying HBC have been made, with particular emphasis on the dog as a model for human cancer. Dogs with intact immune systems spontaneously form mammary tumors and other cancer types, which share certain clinical traits with HBC (Üstün Alkan et al., 2014).

The beginning age, hormonal etiology, and duration of the diseases are just a few of the clinical parallels between mammary cancers in humans and dogs. Tumor size, stage, and lymph node invasion are other characteristics that influence how the disease may manifest. Steroid receptor, epidermal growth factor (EGF), proliferation markers, overexpression of metalloproteinases and cyclooxygenases, and p53 mutation are examples of molecular characteristics (Üstün Alkan et al., 2014).

Positive results when testing the complexes in animals, such as dogs, due to the similarities, as referred above, between the two beings, may indicate that they could also have interesting results when applied to humans (Üstün Alkan et al., 2014). Therefore, the first objective of the thesis would be, after the *in vitro* tests, to move on to *in vivo* tests and possible establishment in the veterinary medicine market, if the results in living beings allow it, aiming to reach the world of human medicine.

1.7. Short summary of the main goals of the thesis

This thesis initial aim was to study human cancer diagnostic and treatment, which, despite years of advancements in this field, continues to be a leading source of morbidity and mortality (Mattiuzzi and Lippi, 2019). Animal models have been crucial in cancer research because human experimenting on humans has both practical and ethical issues. Although cell lines were employed in this study, future research on a compound's effects might move on to studying it in people (Mak et al, 2014).

This thesis underlines two objectives: first, to study the application of new metal complexes forthe treatment of canine mammary cancer, in light of the subject's growing importance and secondly, to discuss a possible technology market strategy for this technology.

Someone unfamiliar with scientific subjects like biotechnology and drug research should be alarmed by the concept of employing a drug that contains a metal because of the possibility of their toxicity in the environment when this sort of treatment is presented. The truth is that many commercialized medications today, not only those used in chemotherapy, have metals in their constitution, being commercialized, so we managed to say that they are not dangerous for the living being.

The experiments were performed at the Human Genetics and Cancer Therapeutics laboratory@UCIBIO coordinated by Professor Alexandra R Fernandes at the Nova School of Science

and Technology, whose is interested in developing novel treatments strategies for human and canine mammary tumors.

1.8. Thesis outline

This thesis is structured in the following way: In this chapter, chapter 1, a introduction was made to all the themes added to the theme of this thesis.

A brief review of the literature on metal complexes and the models used for the study of human cancer is provided in Chapter 2.

Chapter 3 addresses for the experimental work carried out with the various complexes and respective experimental results

Chapter 4 addresses market analysis which includes market dimension and competitors as well as interviews with potential customers (dog owners), prescribers- veterinarians who are going to use the treatment and manufactures as pharmaceutical companies as well as practitioners (veterinarians) and researchers. Also a business model and intellectual property strategy will be presented and discussed.

The conclusion summarizes the main empirical findings as well as the go-to market preferred strategy and also suggests future research that still need to be done.

CHAPTER 2: REVIEW OF THE LITERATURE: METAL COMPLEXES (AND LIGANDS) AND MODELS FOR THE STUDY OF HUMAN CANCER

2.1. Metal complexes

Composts used in medicine nowadays are mostly based on organic compounds. However, there are more and more research addressing the use of metal complexes and their ligands in many medical specialties. Metal ions are special particles that can be used to create novel medications with various modes of action (Karjes, 2020; Ndagi et al, 2017).

Despite making up less than 1% of the atoms in living organisms, metals play a crucial part in biochemical processes and are necessary for the survival of living things. They are frequently present in the catalytic domain of enzymes and are engaged in the exchange of electrons for catalysis or structural functions. Many cellular activities involve the essential metals gallium, zinc, cobalt, silver, vanadium, strontium, manganese, and cooper, whose presence is required in cells in specific concentrations. On the other hand, some metals, such as nickel, cadmium, chromium, and arsenic, can cause carcinogenesis and have less advantageous effects. (Ndagi et al., 2017; Karjes, 2020).

Due to their pharmacological properties, such as their anticancer, anti-inflammatory, antioxidant, and antibacterial activity among others, metal-based medicines have garnered a great deal of attention. Numerous studies have suggested the possibility of an infinite number of therapeutic agents based in metal complexes with various ligands, taking advantage of the properties of metal complexes such as high structural diversity, redox and catalytic properties, and tendency to ligand exchange (Ndagi et al., 2017).

Metal ions and complexes have been used medicinally since the beginning of time, and Barnett Rosenberg's 1960 discovery of cisplatin's anticancer properties provided a springboard for further research in this field (Karjes, 2020; Habala and Valentová, 2018).

The metallic complexes constitute a class of antineoplastic agent of high clinical interest, in which one or more metal ions coordinated to specific ligands play a role a key role in the drug's mechanism of action (Martins et al., 2016).

The toxicity attached to these complexes is the main barrier to their utilization. Most of the time, while exploring a new complex of this sort, the main goal is to develop a molecule with the highest selectivity possible, that is, the biggest difference between these two doses: the therapeutic doses and the hazardous ones are frequently extremely close to one another

The type of ligands, the oxidation state, the geometry of coordination, and the metal at the core of the complex are the variables that may have an impact at this point (Karjes, 2020; Ndagi et al, 2017).

The complexes based on platinum, which are typically used in the market and have undergone extensive research in the battle against cancer, the most important metal complexes and the type of cancer treatment are shown in table 1:

Table 1: Platinum-based complexes: modes of action, disadvantages, and the types of cancer for which they are used.

Complex	Formula	Mechanism of action	Drawbacks	Cancer treatment	References
Cisplatin	$[Pt(NH_3)2Cl_2]$	-Formation of stable DNA adducts that block or inhibit transcription, impacting the development of tumors.	-Nausea -Vomiting -Neuropathy -Myeloid suppression -Ototoxicity -Kidney toxicity -Resistance after 4/6 treatment cycles	-Treatment for various malignancies, with success rates for genital cancers ranging from 70 to 90%, as well as for bladder, head, and neck cancer	(Oun et al, 2018)
Carboplatin	$C_6H_{12}N_2O_4Pt$	-Formation of stable DNA adducts that block or inhibit transcription, impacting the development of tumors.	-Myeloid suppression	-Treatment of various types of cancer (mainly cancer of the lung, ovary, upper digestive tract, and brain)	
Oxaliplatin	$C_8H_{14}N_2O_4Pt$	-Formation of stable DNA adducts that block or inhibit transcription, impacting the development of tumors.	-Numbness -Tiredness -Nausea -Diarrhea -Low blood cell counts -Allergic reactions	-Treatment of lung, ovarian and colon cancer	

The three platinum-based complexes mentioned in the table (Table 1) —cisplatin, carboplatin, and oxaliplatin— are used in around half of chemotherapy treatments, although they have drawbacks include drug resistance, a narrow spectrum of effectiveness, and the potential for adverse effects (Karjes, 2020; Ndagi et al, 2017).

These platinum-based antineoplastics seems to act, by the formation of stable DNA adducts that block or inhibit transcription, impacting the development of tumors (Oun et al, 2018).

Platinum complexes have seen the most advancement, although a great number of other metal complexes with endless combinations of various metals and ligands have also developed. Many of these medications are currently undergoing clinical trials or waiting for ethical approval to be added (Karjes,

2020).

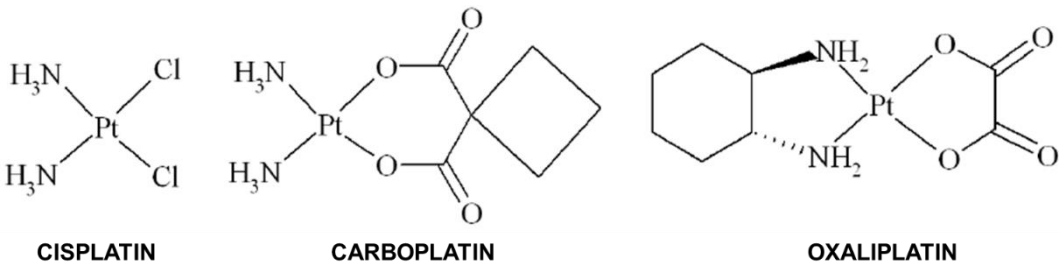

Figure 4: Chemical structure of platinum-based antineoplastics- cisplatin, carboplatin and oxaliplatin (Source: Dospivova et al., 2012))

Platinum medications were quite effective in chemotherapy, but they have limitations. The low solubility and reduced cellular bioavailability of these take to the research for new complexes metals with antineoplastic properties. The objective is to develop a metal-based compound with cytotoxic properties that can overcome all the constraints imposed by platinum compounds (Ndagi et al, 2017).

The last two decades have witnessed the emergence of various metal complexes that do not contain platinum, while it is still theoretically possible and obviously extremely desirable that new platinum compounds appear capable of overcoming the restrictions of cisplatin. With the variation of the metal, consequently, change its chemical properties, has been verified spectrum of therapeutic action, toxicity and mode of action are different from cisplatin compounds (Karjes, 2020; Ndagi et al, 2017).

Many different types of metal complexes, especially transition elements from different places like silver, copper, zinc, iron, ruthenium and vanadiumas well as main group elements like gallium, and bismuth have been synthesized and tested on the last decade (Claudel et al., 2020).The complexe of transition metals have been the main study targets, the partial filling of the d orbital layers give transition metals the electronic properties necessary for the establishment of coordination compounds represented by a metallic center coordinated to a surrounding matrix of molecules or ions (Martins et al., 2016).

Regarding the canine model, most drugs used in the treatment of neoplasms are in the category of organic compounds, such as doxorubicin. There is only one metal complex, cisplatin, among the chemotherapy drugs that are frequently used to treat cancer in canines (Appendix 3).

The mastectomy and ovariohysterectomy are the principal treatments for the bitch's mammary cancer, though. Adjuvant therapy most of the times may be necessary because about half of CMTs are malignant, however medications frequently used to treat human breast cancer have not been demonstrated to significantly benefit canines. Mammary cancer cells have been demonstrated to be exceedingly resistant to the drug cisplatin when used to treat this form of cancer. In the FR37-CMT cell

line, a concentration of up to 50 µM has not been shown to have any appreciable effects (Raposo et al., 2017).

There is great evidence of the need to study and develop new treatments for this morbidity. There is no strong evidence of the use of complexes in CMTs. Data available are the testing of two complexes with DION as a ligand of zinc (TS262) and cobalt (TS265) in the aforementioned cell line, FR37-CMT. These two complexes had already undergone impressive testing in human cancer cell lines (Appendix 7). When testing these compounds in the CMT cell line, these two presented IC50 values in these cells, of approximately 1 µM. This means that the IC50 of these compounds is significantly less than that of cisplatin (50 times lower) and five times less than that of doxorubicin, which are frequently the chemotherapy drugs of choice when chemotherapy is actually necessary (Raposo et al., 2017).

The significant potential of these drugs against CMT is highlighted by the dearth of effective chemotherapeutic treatments for breast cancer in the bitch (Raposo et al., 2017). And it gives us perspectives of new possibilities for compounds to be explored with the same purpose.

2.1.1. Ligands

Metal complexes are made up of metal ions and ligands that are coordinated together. The majority of ligands are neutral or anionic compounds, however cationic compounds are also known but are less prevalent. While anionic ligands are only stabilized when they are coordinated to core metals, neutral ligands are independently stable molecules in their free states (Alcarazo, 2014; Robertson and Cronin, 2002).

2.1.1.1. 1,10-phenanthroline-5,6-dione

The complex's properties and mode of operation will be influenced by the metal in its core, binding it as well. We concentrated on complexes of 1,10-phenanthroline-5,6-dione throughout the laboratory work,either due to its high biological activity, as it has already been cellular and tested in the same composite line with the same interesting results (Raposo et al., 2017). Being the only complexes with this ligand tested in the bitch under study's breast cancer line.

This is a ligand with a phenanthrene base that is a derivative of the traditional chelating agent 1,10-phenanthroline (Calderazzo et al, 2002; McCann et al, 2012). The structure is comparable to that of 1,10-phenanthroline, but it also includes two carbonyl groups linked at positions 5 and 6 (Granato et al , 2017) .

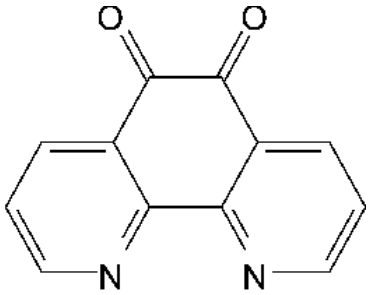

Figure 5: Structure of 1,10-phenanthroline-5,6-dione (Source: (Deegan et al., 2006))

The phenanthroline derivative DION is a bifunctional quinone oxidant that may form stable complexes with a range of metal ions (Wu et al, 1996; Goss and Abruna, 1985; Roy et al, 2008). This one shown its own ability to be cytotoxic against human kidney and hepatocellular carcinomas (Deegan et al,2006; Silva et al, 2013).

This ligand exhibits redox activity as well as the ability to bind with DNA and create covalent connections with proteins (Silva et al, 2013).

Due to its dual functions, redox properties, the presence of two oxygen atoms, and Lewis base behavior brought on by the presence of two diiminic nitrogen atoms, DION is a very flexible ligand with unique reactivity at its quinonoid and diiminic sites (Calderazzo et al, 2002; Calucci et al, 2006). DION is capable of redox reactions thanks to its quinonoid activity, and its two N atoms in close proximity make it the perfect candidate for chelating transition metal ions (Calderazzo et al., 2002; Calucci et al, 2006).

Many intriguing biological characteristics, including anticancer and antibacterial effects, are thought to be present in phendione both in its metal-free state and when coupled to metal ions (McCann et al., 2004, 2012; Deegan et al., 2006; Roy et al., 2008; Pivetta et al., 2014; Viganor et al, 2016).

In order to interact with DNA, phenanthrolines and their metal complexes aromatically stack between base pairs. The helix lengthens, stiffens, and unwinds due to this contact. DION exhibits strong anticancer properties, both in the presence and absence of a coordinated metal (Roy et al,2008).

Many members of this family of compounds, some of which are listed in Appendix 7 as example, even at the HG&CT lab, have already been examined in a variety of cell lines with a wide range of combinations.

2.2. Animal models for the study of human cancer

During the tests carried out within the scope of this, only *in vitro* tests were carried out, in cells, for further studies and possible entry of the compound into the pharmaceutical market, the next steps

would involve testing in living beings (Mak et al, 2014).

Due to practical and ethical concerns associated with human experimentation, animal models have been essential in cancer research.

The discovery and development of any drug is a time-consuming and expensive process. The rising expense of drug research is partially explained by the ineffectiveness of conventional *in vitro* instruments and animal models (Ayuso et al.,2021; Singh et al., 2021).

Traditional cell culture studies commonly fail to predict drug sensitivity, the culture environment does not best mimic the human tumor environment, which is the best explanation for this failure in sensitivity predictions. This barrier is removed by using animal models to research cancer since they can simulate tumor growth in a more pathologic setting (Ayuso et al.,2021; Mendes et al., 2020).

When studying new compounds for oncological treatment in humans, animal models are often used. These have historically played an important role in several studies about the disease, identification of targets and in the evaluation of new therapeutic agents and treatments (Ayuso et al.,2021). As animal models, a variety of animals include fish, birds, reptiles, rabbits, rats, mice, ungulates, and non-human primates can be employed. Researchers must be able to select the species that will best suit the research they are planning by considering the diversity that is accessible (Conn, 2013).

When selecting an animal model, the researcher must have in mind: the purpose of the study, the species available, the advantages and disadvantages of each species, the cost of the animals, maintenance costs, ease of handling, the necessary equipment, and ethical considerations (Fagundes and Taha, 2004).

The difficulties for the study and testing of new compounds, are real, at the level of human medicine, it was then decided to opt for the study of an animal cancer. Choosing an animal model will allow us not only a translational study for the human case, if this present some similitaries with, but it will also give us an opening to approach the veterinary business, which is also an expanding business (Ayuso et al.,2021; Singh et al., 2021).

The understanding of the mechanisms governing the initiation, progression, and metastasis of cancer has greatly benefited from the use of animal models, particularly those of the mouse (representing more than 80% of the animal models used), zebrafish, and drosophila (Figure 6). This understanding has also made it possible to find and preclinically validate new cancer treatments. malignancy, enabling the recreation of tumor growth in a more pathologic setting (Mendes et al., 2020).

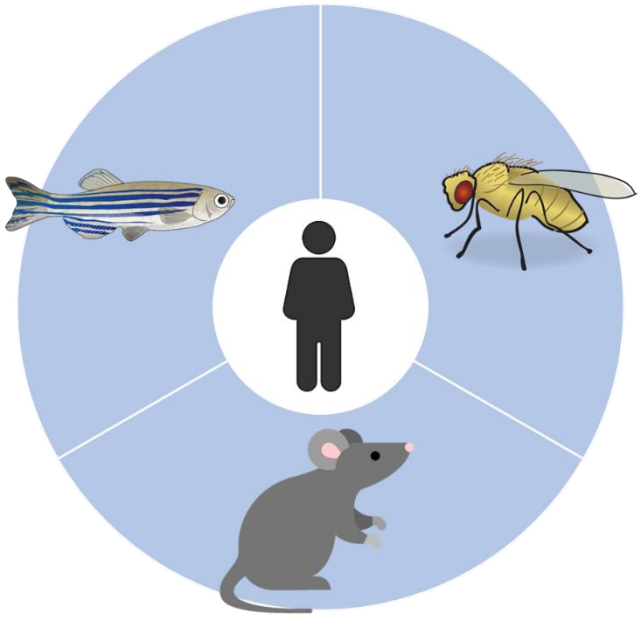

Figure 6: Animal models most used as a model for human cancer (mouse, drosophila and zebrafish)

2.2.1. Rodent Models

Mouse models are great biological models mimetics of physiology, both species follow similar stages of embryonic development and their bodies have the same type complex regulatory bodies and mechanisms. Genomes share a high degree of homology, and there is an overlap in the function of their genes. Furthermore, the mice have a shorter lifespan, allows you to study development and progression of diseases such as cancer in a viable period of time. The economic part, keeping these animals is easy, as is their handling. The use of mice can follow several different approaches, such as Patient Derived Xenografts (Mendes et al., 2020).

Despite being such a well-known model, the mouse's ability to accurately represent human conditions has been questioned due to the apparent lack of correlation between the two organisms. Reproducibility issues in preclinical research have drawn the attention of several researchers. Pre-clinical studies have a significant global economic impact due to the estimated 51–89% irreproducibility of the data presented in clinical trials (DaMatta, 2010; Justice and Dhillon, 2016).

They do not adequately represent several features that define cancer in humans, including long periods of latency, the complex biology of cancer recurrence and metastasis and outcomes to novel therapies (Ranieri et al., 2013).

2.2.2. Zebrafish

Zebrafish is growing in popularity as a versatile *in vivo* model, embryologically and genetically

tractable illness model, for understanding the mechanisms of cancer development and advancing drug discovery because of the optical clarity of embryos and larvae, real-time imaging of growing diseases is possible thanks to zebrafish biology, which provides easy access to all developmental phases, which it uses to supplement traditional studies in mice. The development of the "casper" fish, a transparent adult zebrafish model, has greatly facilitated the analysis of transplanted and endogenous tumors in adult animals. Additionally, embryos and larvae are transparent optics, offering conditions for *in vivo* imaging. Additionally, processes as survival processes of neoplastic cells, angiogenesis, migration, invasion, and metastasis can be better observed due to the transparency of the beings (Lieschke and Currie, 2007; Mendes et al., 2020).

Some data obtained from fish cannot be applied to humans due to cellular differences and their molecular characteristics that are divergent, with fish being a much simpler system than us. Requires more infrastructure and higher maintenance cost compared to other study models (Reis et al., 2017).

2.2.3. Drosophila

This is a model that is pertinent to the study of cancer, according to numerous researches. The fruit fly Drosophila melanogaster has developed into a crucial model system for research on cancer. The characteristics that make Drosophila an important role as a model organism to study the carcinogenesis model include the reduced redundancy in the genome compared to that of humans, conservation in the processes driving the development of cancer between the two species, and the ability to drive genetic screenings by carrying out genetic alterations in specific cells and tissues (Mendes et al., 2020).

Studies are fast compared to those using mammals, and the maintenance cost of fruit flies is low. However, these models are not able to model more complex biological processes and human diseases multifactorial (Pandey and Nichols, 2011).

2.2.4. Dogs

The aforementioned models are extremely simpler than the human model and most of the time they are not subject to the same type of stimuli, which often interfere in the development of the tumor, so when compared, this simplicity shows shortcomings. (Ranieri et al, 2013). Due to the biologic complexity of human cancer, additional models that better investigate the human disease are needed.

Medium-sized animals, such as dogs, are excellent models for experimentation, however, they need elaborate facilities and are expensive (DaMatta, 2010).

In the pet population, companion animals have many desired characteristics that fill the gap

between *in vitro* and *in vivo* studies, and these characteristics have proven to be important in understanding many complex molecular aspects of human cancer. With special references to the dog, cancer is a spontaneous disease and dogs naturally develop cancers that share a wide variety of epidemiologic, biologic, and clinical features with human cancer, which makes this animal model both attractive and underused in oncology research (Ranieri et al, 2013; Pinho et al, 2012).

In terms of nucleotide divergence and rearrangements, dog and human lineages are more comparable than human and rodent lineages. Numerous investigations have revealed a substantial homology for a known cancer-associated gene between dogs and humans (Ranieri et al, 2013).

In relation to breast cancer, more specifically, studies have shown the existence of an important similarity in canine and human genes that were dysregulated at the time of the disease compared to normal samples (Pinho et al., 2012).

The key challenges with this model will be the high expenditures associated with the entire process, the big size of the animals, and the required upkeep when compared to the other models (Kim et al., 2020).

CHAPTER 3: EXPERIMENTAL RESULTS

This chapter presents all the laboratory results on mammary canine cancer treatment with metal complexes under a laboratory environment.

Female dogs provide a good comparative model to understand various aspects of carcinogenesis in both species because the epidemiological, clinical, and biological characteristics of these cancers in female dogs are similar to those in women (Andrade et al., 2010; Üstün Alkan et al., 2014).

Due to the frequent and extensive use of mammography screening and the raised awareness of dog owners, there has been an upsurge in the reported prevalence of HBC and CMT in recent years. The greater sample size for clinical studies in comparative medicine is made possible by the higher incidence of CMT (Üstün Alkan et al., 2014).

3.1. Laboratory experiments

3.1.1. Materials and Methods

3.1.1.1. Cell line and compounds

The cell line used was FR37-CMT, this cell line was immortalized in a previous work in the laboratory, Human Genetics and Cancer Therapeutics, by the Doctor Luís Raposo (Raposo et al, 2017).

This CMT line when compared exhibits greater resistance to doxorubicin and cisplatin than others, exhibits the ability to restructure the collagen matrix, expresses vimentin and CD44, and exhibits the loss of E-cadherin, which is thought to be a crucial step in the epithelial to mesenchymal transition (Raposo et al, 2017).

Different metallic compounds with DION as ligand were tested. Two with a metallic center of manganese (JHOR 9 and F), ruthenium (JHOR10 and JHOR11), iridium (MI12) and vanadium (VO (dipic)(DION)), previously synthesized by Oscar Lenis-Rojas (ITQB) and Katarzyna Choroba, PhD researcher in the group of Dr. Barabra Machura, From the Institute of Chemistry, University of Silesia, Sxkolna Katowice, Poland. For confidentiality reasons, the structures of the compounds will not be shown.

As controls were used cisplatin and doxorubicin. Cisplatin 3 mM stock solution in 0.9% (v/v) NaCl2 (Teva Parenteral Medicines, Teva Pharmaceuticals, Petah Tikva, Israel) and doxorubicin hydrochloride (Sigma, Munich, Germany) 0.4 mM stock solution was made in DMSO (Sigma) and kept at 4°C.

3.1.1.2. Cell culture maintenance

FR37-CMT cell line was grown in an incubator (SANYO CO_2 Incubator, Electric Biomedical Co., Osaka, Japan) maintained in a humidified atmosphere of 5% (v/v) CO_2 and 37 ºC in DMEM supplemented with 10% (v/v) FBS,100 U/mL penicillin and 100 mg/mL streptomycin (Life Technologies). FR37-CMT cell line was maintained in 25 cm^2 vented cell culture flasks (SPL Life Sciences, Korea). Every week cells were subcultured, thus allowing the continued growth, ensuring the necessary nutrients in the medium and avoiding inhibition of growth due to contact. For this purpose, initial medium was discarded and replaced by 2 mL of TrypleTM Express (Invitrogen, New York, EUA), this is a trypsin analogous that allow to detach attached cells from the culture flaks. After 10 min, this action was stopped by adding 1 mL of fresh medium, DMEM. The cell suspension was centrifuged for 5 min at 500 x g (Sigma 3-16K 10280, Tuttlingen, Germany). The supernatant obtained was discarded and the remaining pellet was resuspended in 1 mL of fresh medium. Cells were counted resorting to an hemocytometer (Hirschmann, Eberstadt, Germany) from a mixture of 350 µL of DMEM medium, 100 µL of 0.2% (v/v) trypan blue (Sigma, St. Louis, EUA) and 50 µL of cellular suspension obtained, trypan blue exclusion method. The reagent used, trypan blue, stain cells with compromised membranes being excluded from healthy cells, allowing to count the viable cells. The cells were observed (Olympus CXX41 inverted microscope, Tokyo, Japan) and 1x 10^5 cells//T-flask were seeded in a new T25 flask (Raposo et al,2017).

3.1.1.3. Cell viability assay (MTS)

Cells viability was evaluated by the MTS colorimetric assay, based on the production of a soluble product, formazan, by the intracellular dehydrogenase, enzymes found in active cells in the presence of an electron coupling reagent, as tetrazolium salt, MTS (Stockert, 2018)

FR37-CMT cell line was seeded in 96-well plates (VWR) at a concentration of 0,75x10^5 cells per milliliter and incubated in the conditions described previously. After 24h the media was then removed and replaced with fresh media with the appropriate dilutions of complexes or controls, DMSO and DOX. After 48 h of cell incubation in the presence or absence of compound, cell viability was evaluated through CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay. Briefly, the medium was removed and 100 µL of a solution mixture of fresh medium and MTS reagent (15 µL reagent + 85 µL of medium) was added to each well. Then 96-well plates were incubated during 30 min, protected from light. Afterwards, the absorbance at 490 nm was measured with Tecan Infinite F200 Microplate Reader (Tecan, Männedorf, Switzerland) (Silva et al, 2012).

$$\text{Cell Viability (\%)} = \frac{\text{Samples absorvance (490 nm)} - \text{Complex absorvance (490 nm)}}{\text{DMSO absorvance (490 nm)} - \text{Medium absorvance (490 nm)}} \times 100$$

Equation 1- Calculation of the percentage of viable cells present in the sample in analysis

The methodology allows to determine the number of viable cells (Equation 1), taking account that the amount of formazan measured in the assay is directly proportional to the number of living cells in a culture (Wang et al., 2010). The relative IC_{50}, compound concentration that causes 50% of cell metabolic inhibition, was calculated from dose-response curves resorting to GraphPadPrism 8 Software, with a confidence interval of 95% (Neubig et al. 2003).

3.1.1.4. Annexin V-FITC and PI double stanning assay (Apoptosis)

The annexin V-binding assay using flow cytometry is used to detect and distinguish between early apoptosis and late apoptosis, as well as necrosis (Henry et al., 2013).

FR37- CMT cells were seeded in 6-well plates (VWR, Europe) at $1x10^5$ cells/mL and incubated in the conditions described previously. Culture medium was removed after 24 h and replaced with 2 mL of fresh medium containing either IC_{50} compound concentration, 0.1% (v/v) DMSO (vehicle control), 5 µM of CIS or 0.4 µM of DOX. Cells were incubated for 48 h, collected by trypsinization and centrifuged for 5 min at 500 x g (Sigma 3-16K 10280, Tuttlingen, Germany). The obtained pellet was rinsed twice with 1 mL of PBS 1x intercalated with 5 min of centrifugations at 750 x g. Following centrifugations, 100 µL of annexin binding buffer 1x, 5 µL of annexin V-FITC and 1 µL of PI (Annexin V-FITC Apoptosis Detection Kit; Invitrogen, USA) was added to all samples and incubated for 15 min in the absence of light. Afterwards, to these cellular suspensions were added 400 µL of annexin binding buffer 1x. Cells were analyzed using an Attune ® Acoustic Focusing Flow Cytometer (ThermoFisher Scientific, Waltham,MA, USA) with the acquisition of at least 10 000 events per sample. Data presented here are from at least two independent experiments.

3.1.1.5. Assessment to autophagic potential

Autophagy was studied using the Autophagy Assay Kit (Abcam, Cambridge, UK). The detection reagent used becomes fluorescent in vesicles produced during autophagy allowing quantification of autophagic cells.

FR37- CMT cells were seeded in 6-well plates (VWR, Europe) at $1x10^5$ cells/mL and incubated in the conditions described previously. Culture medium was removed after 24 h and replaced with 2 mL of fresh medium containing either IC_{50} compound concentration, 0.1% (v/v) DMSO (vehicle control), 5 µM of CIS or 0.4 µM of DOX. 15 h before the ending of the 48h of incubation, 0.5 µM rapamycin, an autophagy inductor, was added to the corresponding well. At the end of the 48h incubated, Cells were collected with trypsin, washed with Assay Buffer 1 ×, and incubated in DMEM culture medium with Green Stain solution and 5% (v/v) FBS for 30 min. Cells were then collected and washed with Assay Buffer 1x. Samples were resuspended in Assay Buffer 1 × and analyzed using an Attune ® Acoustic Focusing Flow Cytometer (ThermoFisher Scientific, Waltham,MA, USA) with the acquisition of at least 10 000 events per sample. Data presented here are from at least two independent experiments.

3.1.1.6. H2DCF-dA staining assay (Measurement of production of intracellular ROS)

Reactive oxygen species (ROS) Detection Reagents (Life Technologies, InvitrogenTM, USA) let us detect the accumulation of intracellular ROS. The reagent used, H2DCF-dA, when oxidized by peroxides is converted into a high fluorescent component, 2,7-dichloroflurescein, directly proportional to amount of ROS in cells (Marchi et al. 2012).

For the H2DCF-dA staining assay, FR37-CMT cells were in 6-well plates (VWR, Europe) at $1x10^5$ cells/mL and incubated in the conditions described previously. After the incubation period, the medium was removed and replaced by fresh medium containing IC_{50} of compounds, 0.1% (v/v) DMSO (Negative control solvent), 18.1 µM of TBHP(positive control), 5 µM of CIS or 0.4 µM of DOX (Positive control). After 48 h of exposure cells were washed with PBS 1x and stained with 100 mM of H2DCF-dA in PBS 1x and incubated for 20 min at 37°C, protected from light. Cells were analyzed using an Attune ® Acoustic Focusing Flow Cytometer (ThermoFisher Scientific, Waltham,MA, USA) with the acquisition of at least 10 000 events per sample. Data presented here are from at least two independent experiments.

3.1.1.7. Measurement of mitochondrial membrane potential ($\Delta\boldsymbol{\Psi M}$)

The mitochondrial membrane potential (ΔΨM) shows us if mitochondria are functioning or not, being an indicator of cell health (Christensen et al., 2013). To measure ΔΨM JC-1 (Abnova Corporation, Walnut, CA, USA) was used, a lipophilic cationic dye.

FR37- CMT cells were seeded in 6-well plates (VWR, Europe) at $1x10^5$ cells/mL and incubated in the conditions described previously. Culture medium was removed after 24 h and replaced with 2 mL of fresh medium containing either IC_{50} compound concentration, 0.1% (v/v) DMSO (vehicle control), 5 µM of CIS or 0.4 µM of DOX. Cells were incubated for 48 h and later collected, washed with PBS 1 ×, and incubated with JC-1 probe for 20 min. Afterward, cells were collected, resuspended in PBS 1 ×, and analyzed using an Attune ® Acoustic Focusing Flow Cytometer (ThermoFisher Scientific, Waltham,MA, USA) with the acquisition of at least 10 000 events per sample. Data presented here are from at least two independent experiments.

3.1.1.8. Wound healing assay or cell migration assay

FR37-CMT cell line was seeded in 24-well plates (VWR) at a concentration of $2x10^5$ cells per milliliter and incubated with controlled atmosphere, 5% (v/v) CO_2 and 99% (v/v) humidity at 37°C. After 24h hours a scratch was made with a sterile 100 µL micropipette tip on the surface of the wells. Cells were

then exposed to IC_{50} of compounds, 0.1% (v/v) DMSO (vehicle control) or 0.4 µM of DOX and incubated for 24h in the same conditions describe previously. Cell cultures were photographed, and the remission percentage was calculated by measuring the width of scratches with the use of ImageJ 1.49v software. Data presented here are from two independent experiments (Raposo et al., 2017).

3.1.1.9. Statistical analysis

All data were expressed as mean ± SEM from at least two independent experiments. Statistical analysis was performed using GraphPad Prism v.8.0.1 through One or Two-way ANOVA for comparing the control group with treatment groups for statistical significance. Statistical significance was considered when p-value < 0.05.

3.1.2. Results

3.1.2.1. Cell viability assay

The cytotoxic potential of two substances containing the DION ligand, TS262 and TS265, was examined in earlier research conducted by Raposo et al., 2017, in this cell line, FR37-CMT. In light of this, during the current experiment, the antiproliferative activity of additional compounds from this family was examined in this cell line at doses ranging from 0.01 to 50 µM with a 48-hour exposure time.

The *in vitro* antiproliferative effect of the six complexes were analyzed in the canine mammary cell line, FR37-CMT, to identify which metal complex induces a more pronounced growth inhibition (Figure 7).

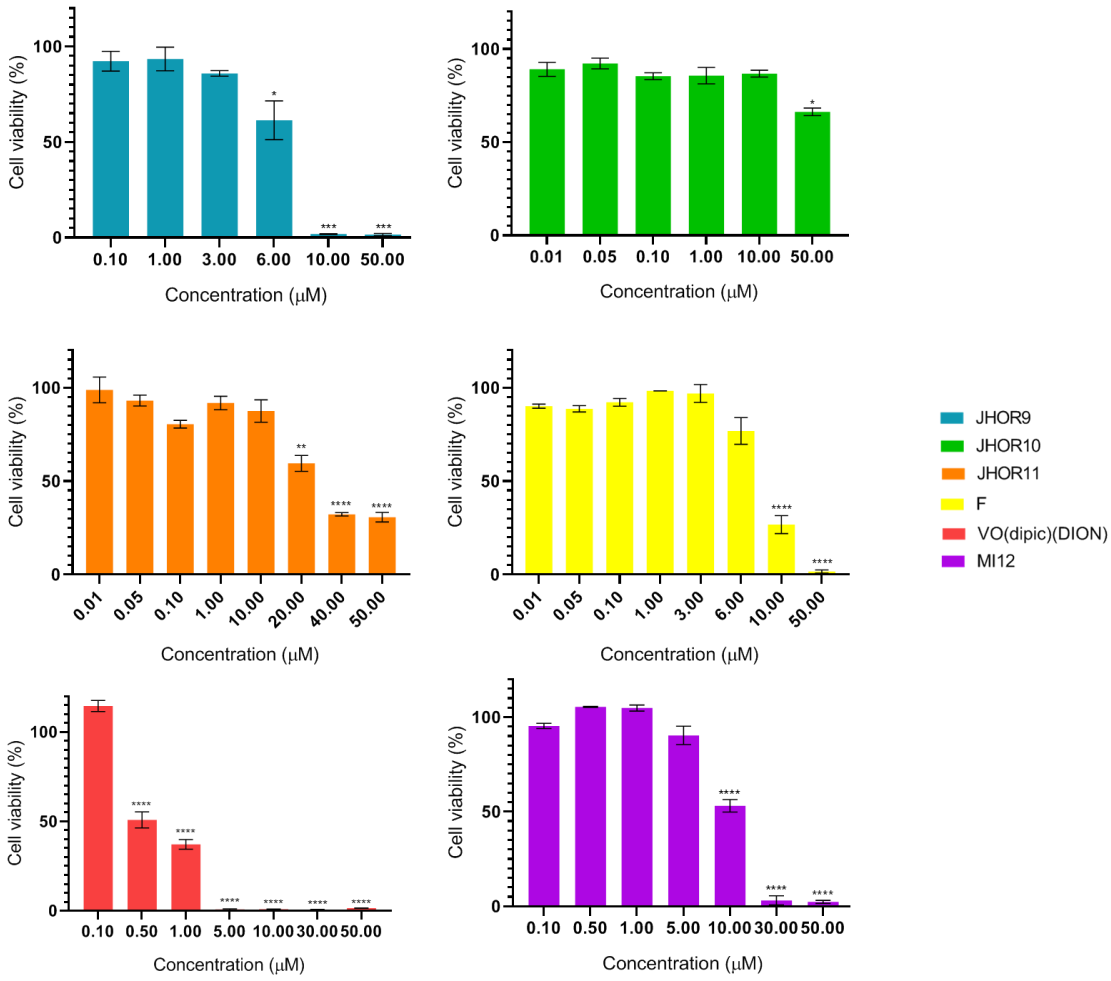

Figure 7: Cell viability (%) of FR37-CMT cells after 48 hours of exposure to different concentrations of complexes. DMSO 0.1% (v/v) was used as vehicle control. Represented data are the mean ± SEM of two independent experiments (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Relative IC50 was calculated from dose-response curves resorting to the GraphPad Prism 8 software.

Based on the results presented in Figure 4, the respective IC_{50} of the compounds were calculated using the GraphPadPrism 8 Software (Table 2). The selectivity index (SI) was calculated for FR37-CMT cell line compared to fibroblasts (Table 3).

$$Seletivity\ index\ (SI) = \frac{IC_{50}\ in\ fibroblasts}{IC_{50}\ in\ FR37-CMT}$$

Equation 2: Calculation of the selectivity index of each tested compound

Figure 4 shows a decrease in cell viability after 48 h of exposure to increasing concentrations of JHOR9, JHOR11, F, VO(dipic)(DION) and MI12 complexes. Complex JHOR10 does not seem to induce cytotoxicity in tumor or normal cells with IC_{50} higher than 50 μM (Table 2)

Table 2: Values for the relative IC_{50} of FR37-CMT after 48 h exposure to JHOR9, JHOR10, JHOR11, F, VO(dipic)(DION) and MI12 obtained using the GraphPadPrism 8 Software. Concentrations are expressed in μM.

	Relative IC_{50} (μM)					
	JHOR9	JHOR10	JHOR11	F	VO(dipic)(DION)	MI12
FR37-CMT	6.41	> 50	19.80	8.20	0.28	10.22

For the FR37-CMT cancer cell line, the IC_{50}, of the regularly used anticancer medicines, cisplatin and doxorubicin, is greater than 50 μM and 5.3 μM, respectively (Raposo et al.,2017).

The cytotoxic potential is more pronounced for VO(dipic)(DION) complex with an IC_{50} concentration value in canine mammary cancer cell line, FR37-CMT, of 0.28 μM. After this compound, the compounds with the highest cytotoxicity are the manganese complexes, JHOR9 and F, with an IC50 lower than 10 μM. In fact, complexes JHOR9 and F are structurally similar, with complex F having one additional acridine ligand compared to complex JHOR9.

Complexes JHOR9 and F have an antiproliferative effect in FR37-CMT in the same order of magnitude of doxorubicin and higher than cisplatin. As for the vanadium complex, it has greater antiproliferative effect in this cells higher than that to chemotherapics, DOX and CIS. This may indicate that these three complexes may represent alternatives to these known drugs.

The selectivity index (SI) in all complexes are very low, close to the unit , thus not being able to affirm that there is selectivity for the tumor cells. The SI results should present values greater than 5/10. Despite all this, when choosing a complex for a more effective treatment for this cancer, the best would be vanadium, as the therapeutic margin is greater (> SI). It can then be said that this is the compound with the highest antiproliferative and most selective potential among all the compounds of this family studied in this cell line so far. To calculate the SI values, data from literature on IC_{50} in fibroblasts of the compounds under study were used (Table 3) (Lenis-Rojas, 2021; Raposo et al, 2017).

The values presented in fibroblasts demonstrate that these compounds can be very cytotoxic in normal cells. For that reason in previous studies, the compounds TS262 and TS265 were nanovectorized in order to be more selective. This nanovectorization increased the antiproliferative power of these compounds (Raposo et al, 2017).

Table 3: Values of selectivity (IC_{50} of Fibroblasts /IC_{50} FR37-CMT ratio) of JHOR9, F and VO(dipic)(DION) and previously tested DION compounds, TS262 and TS265.

IC50 (μM)	**FR37-CMT**	**Fibroblasts**	**Selectivity index (SI)**
TS262	1.05	0.6	0.57
TS265	1.39	0.62	0.44
JHOR9	6.41	8.4	1.31
F	8.2	8.9	1.09
VO(dipic)(DION)	0.28	0.65	2.29

The other compounds of this family have been previously tested, TS262 and TS265 *in vitro* and *in vivo* and demonstrated that they were not able to induce systemic toxicity in mouse xenografts, and do not accumulate in the liver and brain of the mice but instead, the treatment is targeted to the tumor (Fernandes et al., 2017; Pedrosa et al., 2019). However, as can be seen from Table 5, these the complexes under evaluation in this work present a higher selectivity compared with the two former.

The vanadium complex, in addition of being the complex that demonstrated the lower IC_{50} in FR37-CMT cells, also demonstrates a higher selectivity for the mammary tumor cell line when compared to normal cells, human fibroblasts (Table 3). it would have been interesting, for this work, to compare in a model of normal bitch epithelial cells and not using human fibroblasts as a comparison due to different species-specific sensitivities.

By showing a greater cytotoxic effect in FR37-CMT cell line and better selectivity compared to the previous studied complexes (TS262 and TS265) (Fernandes et al., 2017; Pedrosa et al., 2019; Raposo et al., 2019) the manganese and vanadium complexes were chosen for the further biologic assays and characterization of the mechanism underlying the loss of cell viability above observed (Figure 7 and Table 4).

3.1.2.2. Apoptosis study

To discriminate between necrotic and apoptotic cell death, double labeling with annexin V-FITC and PI was utilized. The physical traits and metabolic alterations allow for simple differentiation. Phosphatidylserine is translocated to the outer membrane when apoptosis occurs, allowing the annexin to bind the PS (Brumatti et al., 2008). While late stages of apoptosis and necrosis alter the integrity of cell membranes, allowing the PI to reach the nucleus and attach to DNA molecules (Brun et al. 2012). It is feasible to identify and count cells using this staining by flow cytometry (Henry et al., 2013):

- Viable cells: Annexin V (-) and PI (-)
- Early apoptotic cells: Annexin V (+) and PI (-)
- Late apoptotic cells: Annexin V (+) and PI (+)
- Necrotic cells: Annexin V (-) and PI (+)

When FR37-CMT is exposed to JHOR9 and VO(dipic)(DION), a small but not statistically significant increase in the percentage of apoptotic cells is observed (early and late apotosis) compared to the vehicle control, DMSO (Figure 8). The fraction of necrotic cells remained very low with complex exposure (Figure 8).

On the other hand, the percentage of apoptotic cells after exposure to the concentration of IC_{50} of the manganese compound F shows to be the same as when the cells were exposed to the vehicle

control, DMSO (Figure 8).

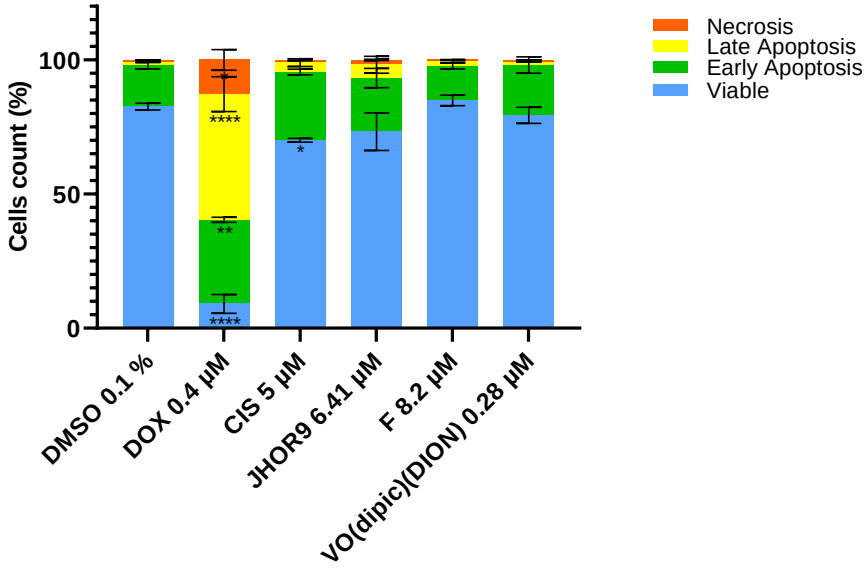

Figure 8: Evaluation and quantification of apoptosis and necrosis by flow cytometry with annexin V-FITC and PI double staining. FR37-CMT cells after 48 h of exposure to IC50 concentrations of JHOR9, F and VO(dipic)(DION). 0.1% (v/v) DMSO was used as vehicle control and 5 µM cisplatin and 0.4 µM doxorubicin as positive control. Data are presented as the mean ± SEM and statistics was performed by Two-way ANOVA compared to control of at least two independent experiments using the GraphPad Prism 8 software. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

These results seem to indicate that when FR37-CMT cells are exposed to these complexes, apoptosis is not trigger and other cell death mechanism might be responsible for loss of cell viability observed above (Figures 7 and 8). To further confirm these results the measure of mitochondrial membrane potential was accessed.

3.1.2.3. Measurement of mitochondrial membrane potential ($\Delta\Psi M$)

Changes in the ΔΨM in cells treated with the different compounds were analyzed to check the functional activity of the mitochondria. JC- 1 dye is lipophilic charged positively (naturally exhibiting green fluorescence) which spontaneously accumulates in the matrix of mitochondria in healthy cells due to the negative potential of the inner mitochondrial membrane, start forming reversible aggregates. These aggregates exhibit fluorescence in the red spectrum. In healthy cells with normal ΔΨM, the red fluorescent aggregates were formed. By contrast, in unhealthy cells or apoptotic, due to the increase of membrane permeability and consequent loss of electrochemical potential, in this conditions JC-1 does not reach a sufficient concentration to form aggregates, remaining in its monomeric form (green fluorescence). So in presence of unhealthy cells, with low ΔΨM, were observed the loss of red fluorescence and an increase in cytoplasmic green fluorescence cause the ΔΨM to decrease, making it a valuable measure of the health and functional condition of cells (Perelman et al. 2012; Sivandzade, 2019). Cells were analyzed by flow cytometry and results are shown in Figure 9.

As expected, based on the previous apoptotic results (Figure 5) no induction of depolarization of mitochondrial potential is observed for the tested complexes (Figure 6) as the ratio between monomeric and aggregated form are similar (no statistically significant results). Moreover, the concentrations used for the two common antitumor drugs, doxorubicin and cisplatin, contrary to what is seen in human cell lines (Raposo et al., 2017) do not seem to trigger mitochondria dependent apoptosis (Figure 8 and Figure 9).

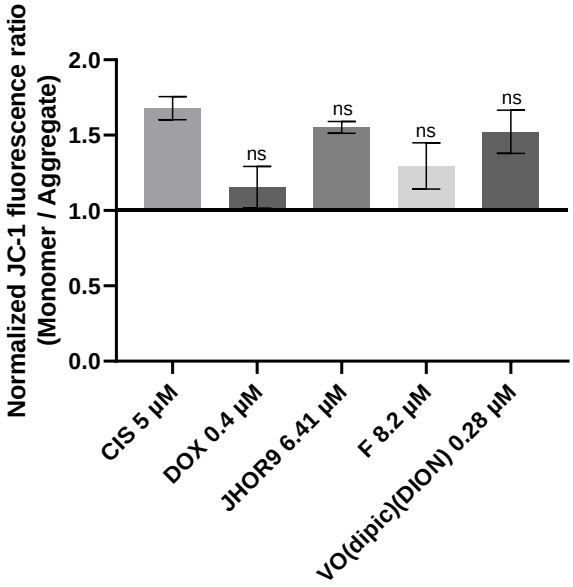

Figure 9: JC-1 monomer/aggregate fluorescence ratio in FR37-CMT cells after 48 h of exposure to IC_{50} concentrations of JHOR9, F and VO(dipic)(DION). 0.1% (v/v) DMSO was used as vehicle control and 5 µM cisplatin ,0.4 µM doxorubicin and 18.1 µM TBHP as positive control. Data are presented as the mean ± SEM and statistics was performed by One-way ANOVA compared to control of at least two independent experiments using the GraphPad Prism 8 software. (ns - statistically non-significant).

Figure 9 shows that exposure of FR37-CMT to the different complexes induced permeabilization of the mitochondrial membrane (ratio > 1). Despite ratios greater than 1, none of the results with the three different complexes was statistically significant. With these results, therefore, we cannot confirm that the loss of cell viability after exposure to IC_{50} concentrations is correlated with the triggering the intrinsic pathway of apoptosis.

Based on these results other cell death, autophagic cell death, mechanism was assessed.

3.1.2.4. Assessment to autophagic potential

To determine if JHOR9, F, and VO(dipic)(DION) also produce autophagic cell death in addition to apoptosis, the assessment of autophagic cell death was also examined.

Cyto-ID® Green dye, a 488nm-excitable green, fluorescent reagent that becomes intensely luminous in vesicles created during autophagy and can be recognized by flow cytometry, was used in

the experiment for this purpose (Guo, 2015).

Compared to the control group, the complexes-treated cells showed a substantial increase in autophagy (Figure 10). The manganese compounds tested showed a percentage of autophagy 3.4 times higher than the negative control and the slightly higher vanadium complex, 3.7 times higher. When our compounds were compared with the positive control (rapamycin) and the antitumor drugs, we found that the percentage of autophagy observed when exposed to IC50 concentrations of the compounds is very close to, between 0.7 and 0.9 times, the percentage of cells in autophagy recorded in the positive control and the known drugs (Figure 10).

These results suggest that exposure of FR37-CMT cells to both compounds can induce a non-apoptotic cell death in cancer cells such as autophagy.

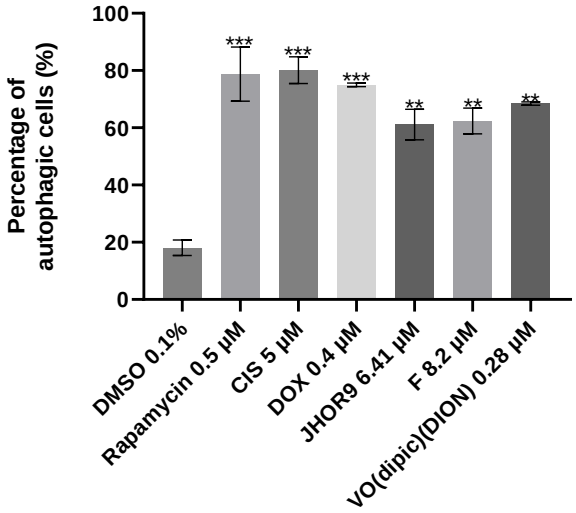

Figure 10: Induction of autophagy after exposure of FR37-CMT cells after 48 h of exposure to IC_{50} concentrations of JHOR9, F and VO(dipic)(DION). 0.1% (v/v) DMSO was used as vehicle control and 5 µM cisplatin ,0.4 µM doxorubicin and 0.5 µM rapamycin as positive control. Data are presented as the mean ± SEM and statistics was performed by One-way ANOVA compared to control of at least two independent experiments using the GraphPad Prism 8 software. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

The autophagic process, a form of programmed cell death, is commonly induced during cancer treatments, such as chemotherapy (Rahmati et al., 2020).

To further understand the reason for this loss of cell viability via autophagy and knowing that metal complexes are known to induce oxidative stress, we accessed the production of reactive oxygen species in FR37-CMT cells exposed for 48h to the complexes.

3.1.2.5. Measurement of production of intracellular reactive oxygen species

Metal complexes are known to cause cell death by producing ROS, and in this regard, FR37-CMT cells were exposed to the IC_{50} of each complex for 48 hours. Following that exposure time, intracellular ROS levels were measured using H2DCF-dA, a cell-permeable, non-fluorescent substance

that accumulates inside of cells upon de-acetylation and interacts with ROS to produce highly fluorescent molecules (McKenzie et al., 2017; TungáLeung et al., 2014) (Figure 11).

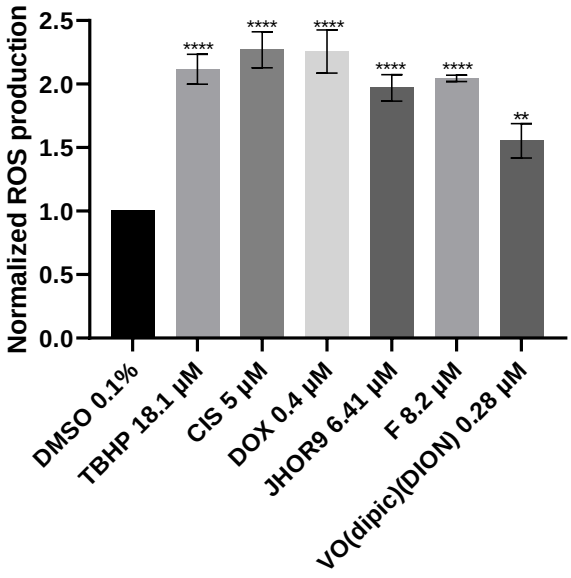

Figure 11: Reactive oxygen species (ROS) induced in FR37-CMT cells after 48 h of exposure to IC50 concentrations of JHOR9, F and VO(dipic)(DION). 0.1% (v/v) DMSO was used as vehicle control and 5 µM cisplatin ,0.4 µM doxorubicin and 18.1 µM TBHP as positive control. Data are presented as the mean ± SEM and statistics was performed by One-way ANOVA compared to control of at least two independent experiments using the GraphPad Prism 8 software. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

An increase in ROS levels was observed in cells treated with the three metal complexes when compared to control cells (Figure 11). Moreover, cells treated with the manganese complexes (JHOR9 and F) produced 2x the level of ROS while cells treated with the vanadium complex a 1.5x ROS levels (Figure 11) Interestingly, the manganese complexes produced levels of ROS similar to the ones produced by the positive control TBHP or the common antitumor drugs, cisplatin and doxorubicin (Figure 11).

The complexes cause FR37-CMT cells to experience an increased oxidative stress, which triggers autophagic programmed cell death mechanisms above observed (Figure 10). These results also agree with the literature and with the oxidative stress induced by manganese and vanadium complexes (Kowalski, 2020; Lenis‐Rojas et al., 2021).

3.1.2.6. Wound healing assay or cell migration assay

Researchers have used cell migration assay for many years to investigate cell polarization, remodel the tissue matrix, or calculate the rates of cell migration and proliferation in various cell types and culture environments. This "wound" leaves an open gap that is later examined under a microscope as the cells fill the wound over time. Depending on the type of cell, the environment, and the surface

area, or space between cells, the "healing" effect can take several hours to many days. The different shapes and sizes of the wounds prevent consistent outcomes and lead to fluctuation from one well to another. Additionally, "the scratch wound" experiment frequently results in cell damage around the wound's edge, which might obstruct cell migration into the wound site and hinder healing. (Trepat, 2012)

Previously, data from our lab has demonstrated that FR37-CMT cells have a strong migration capability which is in line with the loss of E-cadherin expression, which is essential for controlling epithelial-mesenchymal transition (Raposo et al., 2017).

As observed in Figure 12, all complexes can reduce the migration of cells leading to a lower percentage of remission compared to control vehicle. The percentage of remission is F> VO(dipic)(DION) ≅ Doxorubicin > JHOR9, meaning that JHOR9 can sustain FR37-CMT cell migration in a higher extend (Figures 12). Interestingly, JHOR9 shows an ability to interfere with FR37-CMT migration in a higher extent compared to the antitumor drug doxorubicin (Figure 12).

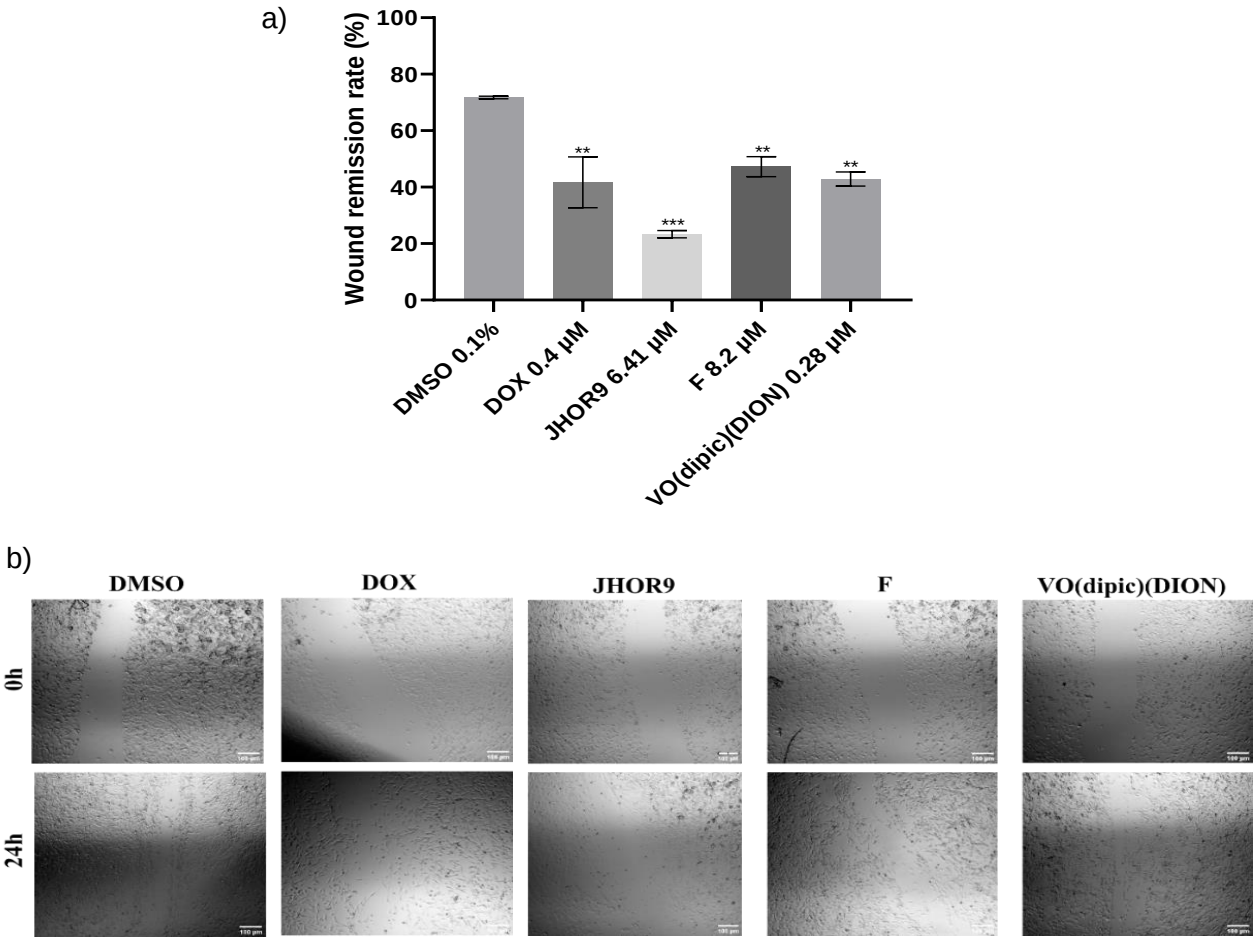

Figure 12: a) Migration assay of FR37-CMT cells exposed to IC50 concentrations of JHOR9, F and VO(dipic)(DION). 0.1% (v/v) DMSO was used as vehicle control and 0.4 µM doxorubicin as positive control. The remission rates were calculated by measuring scratches at time 0h and after 24h of exposition. Data are presented as the mean ± SEM and statistics was performed by One-way ANOVA compared to control of two independent experiments using the GraphPad Prism 8 software. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). b) Representative images of wound healing assay at 0h and 24h of exposure to the IC50 concentrations of JHOR9, F and VO(dipic)(DION), 0.1% (v/v) DMSO and 0.4 µM doxorubicin.

The manganese compound, JHOR9, which has a wound remission rate around four times lower compared to the control (DMSO), has the most influence on cell migration and matrix remodeling ability, according to the findings of this study (Figure 12).

3.2. Final Remarks

These experiments were conducted to determine which complex would be most suited to continue the required *in vivo* testing and, perhaps, with interest to the veterinary pharmaceutical industry's.

It would be interesting to proceed with one of these compounds for future tests. But which is the most interesting compound to proceed with testing? In order to answer this question in a systematized

way this thesis uses the Value Creation Wheel (VCW) methodology (Lages et al, 2020; Lages, 2016). The Value Creation Wheel (VCW) is a decision-making meta-framework that helps to identify, analyse, and solve problems recommended to face all kinds of challenges. This framework allows the creation of value for society and all stakeholders involved in the value chain. In this session is going to be discuss the most appropriate metal complex to proceed testes for the treatment of cancer treatment. All the compounds of the family of compounds with DION as ligand previously tested in FR37-CMT cell line were considered and made as well as several criteria that should be considered for choosing the specific complex.

In the application of this VCW, eliminatory filters will not be applied, but copulative filters. That is, instead of eliminating a complex that does not satisfy the predispositions of a filter, it will receive points each time it fulfills one of the requirements. The selected complex will be the one with the most points after applying all the filters.

The filters chosen were for this VCW: cytotoxicity, selectivity, possibility of patentability and *in vivo* tests. These four filters are then ranked from 1^{s}t to 4th, in the order they are presented.

Cytotoxicity (1st) will refer to the results obtained during the viability tests performed, complexes with IC_{50} lower than 10 µM, respect this requirement, as well as selectivity (2nd), which refers to the comparison between the IC50 doses between tumor cells, FR37-CMT, and fibroblasts. The patentability filter (3rd) this filter will select the complexes that are subject to patent, that is, the complexes which there are no publications or published information about them, fulfilling the patentability requirement with regard to intellectual property, this prerequisite will be explored more in the next chapter. As for the last filter, we have the *in vivo* results tests (4 th), the complexes that fulfilled this requirement will be the ones that already have good results in previous tests in live models.

As we can see none of the compounds meets all these assumptions. The only compound that fufill 3 out of 4 criteria (Table 4) was the vanadium complex. This one meets the requirements of cytotoxicity, selectivity (having been the only one to "scored" in this requirement due to the low selectivity of all the complexes, this being the one with the best results) and possibility of patentability. The single requirement that this chemical is that we didn't know how it behaves in live models, *in vivo* experiments have not yet been performed with this.

Table 4: Value creation Funnel on the DION complexes tested in the laboratory in FR37-CMT cell line. With 1 is marked when satisfies a requirement, with 0 when it does not and with (-) when something has not been tested yet.

	Citotoxicity	**Selectivity**	**Patenteability**	***In vivo* results**
JHOR9	1	0	0	1
JHOR10	0	0	0	-
JHOR11	0	0	0	-
F	1	0	0	1
MI12	0	0	1	-
VO(dipic)(DION)	1	1	1	-
TS262	1	0	0	1
TS265	1	0	0	1

The only complexes that were previously tested in an *in vivo* model were the manganese ones, JHOR 9 and F, and the TS262 and TS265 complexes. These last two were tested in murine models, while manganese were tested in xenograph models in zebrafish, others in mouse models and in embryos. All these did not show systemic toxicity in the presence of high concentrations of the complexes (Fernandes, 2017; Lenis-Rojas,2022; Pedrosa, 2019).

However, it can be confirmed that none of the other compounds analized have undergone *in vivo* testing. It is anticipated that it will function in a manner consistent with its documented systemic toxicity, as studied in zebrafish, mouse models, and embryos (Lenis-Rojas,2022; Raposo et al., 2017).

After these *in vitro* testing, the goal is to go on to *in vivo* studies and if possible towards clinical validation in dogs, and VO(dipic) (DION) complex seems to be the complex that would be most appealing for those studies.

We may be interesting to use nanotechnology techniques to create structures to vectorize the compound (Raposo et al 2017). In this regard, if it turns out during the *in vivo* tests that the vanadium complex is not specific for the tumor and accumulates in other organs, such as the brain and liver, targeted delivery, and increasing drug bioavailability with less side effects, might prompt to a better therapeutic efficacy (Freitas et al., 2011). This branch of study has experienced rapid advancement in recent years and has proven to be quite effective in resolving issues of this nature (Fernandes, 2017; Freitas and Muniz, 2020; Pedrosa, 2019).

The next chapter will discuss technology market transfer strategy. A key element is the Intellectual Property strategy which is also going to be addressed in Chapter 4.

CHAPTER 4: TECHNOLOGY MARKET TRANSFER STRATEGY

Intellectual Property Rights should be analysed in before commercialization of a breakthrough technology. It may help increase the chances of commercial success.

4.1. Intellectual Property Protection

Inventions can be protected by a patent or trade secret. This strategy creates a barrier against market competitors which may foster businesses to innovate.

Patents are intangible assets that are important in creating value, increasingly important, which are sources of value in the most important competitive advantages. But what is this intangible asset? They are considered as rights to future benefits that do not materialize physically or financially (Gu and Lev, 2001).

Identification and preservation of intellectual property assets reveal to be one of the key external aspects in a company's strategy to generate value in the health/biotechnology sector, where there is continual need for scientific and technical innovation, being crucial for the success of investment choices and use of a specific technology. Following the product's research and development phase, it is determined to investigate create a sufficient property management strategy for commercial intellectual properties that ensures us have a commercial edge in canine oncology. But the obstacle in this industry, it is difficult and highly demanding to secure these rights to a corporation (Krejcar, 2020).

There are a few conditions that must be met before an invention can be patented; these include that it must not yet be protected, not yet be known to the public, and not yet have been advertised or described by the inventor, the applicant, or anyone else in Portugal or any other nation.

In this work, the potential of six complexes in canine cancer cells was investigated and two more of these complexes had previously been tested. The truth is that of these, in total eight complexes, only two would be eligible to be patented, the six others having already been published, being then of public knowledge. The complexes that could be patented are MI12 and VO(dipic)(DION).

The patent will be a benefit in the acquisition of value for this technology. This procedure takes both time and money, and the complexity of the entire process might vary depending on the entities involved.

European legislation on patents enshrines the principle of non-patentability of medical or surgical treatment methods for people or animals (Sterckx, 2010).Therefore, the treatment with the

complex in question would not be patentable, in this case what would be the target of protection would be the structure of the complex.

In a possible intellectual property process with one of the two possible complexes, there would be some differences in the all process between them. The iridium complex is a complex synthesized in an institution, ITQB, belonging to the university to which the FCT is also part, the UNL, which would facilitate the entire process. On the other hand, we have the vanadium complex, which obtained better results in the *in vitro* tests, as it was possible to verify in the previous chapter, this was synthesized by a faculty outside the UNL, even outside Portugal, in a university in Poland, a patent of this it would then involve two institutions and even two different countries, possibly with possible regulations, which could complicate and delay the whole process.

In the process of patenting the vanadium compound, the two institutions would need to be involved, the University of Silesia, Sxkolna Katowice, in Poland, for being responsible for the synthesis of the compound and the FCT, more specifically the HG&CT laboratory, for the results and for the entire process of testing the compound in cell lines.

4.2. Regulation

In terms of regulation for testing on animals, for clinical trials, is increasingly demanding over the years, these must ensure both their physical well-being and the efficacy and safety of veterinary medications. These can only be carried out by qualified technicians with the necessary scientific training, research experience, and expertise, particularly in the area of testing clinical proposals, under veterinary supervision. They can only be done at locations with the equipment and staff necessary to provide the level of scientific rigor and excellence required (Appendix 4).

The DGAV and INFARMED must approve clinical studies before they can be conducted. The protocol and other technical-scientific material that supports the goals of the clinical trial and specifies the veterinary medicine being tested must be included with the authorization request, which should be sent to the director general of Veterinary (Appendix 4).

The National Institute of Pharmacy and Medicine, often known as INFARMED, must give its approval before veterinary medical items can be sold (Coelho, 2013).

Based on information in DGAV's site, in Portugal, experimental projects or any use that involves the use of animals, must be under cover Decree-Law n.º 113/2013 , must be previously evaluated and authorized by the DGAV and, for that, they must go through a Project Authorization Process with it. To request a Project Authorization, it is necessary to provide documentation with the same: application requesting project authorization, a form for requesting a Project Authorization, Non-binding opinion issued by the Agency Responsible for Animal Welfare, Non-technical Abstract Template of project,

together with the respective payment and proof.

Authorization request form for the use of animals project for scientific purposes is divided into six sections: administrative details about the project, people and establishments involved, information about the project, objectives and potential benefits of the project, information about the animals, information about the procedures and experimental design and declaration of responsibilities.

As for the non-technical summary model of the project, it consists of an excel, where the main information of the project is placed, such as title and language, project purposes, expected damages and destination of the live animals.

Next session addresses market analysis which includes market dimension and the following one discusses the competitors.

4.3. Market Research and Analysis

The value of market knowledge is comparable to a study that involves gathering and utilizing data as a crucial decision-making tool, enabling discussion of the viability of upcoming endeavors or enterprises.

The market for veterinary oncology worldwide, which was estimated to be worth US$297.30 million in 2020, is anticipated to expand to US$819.84 million by 2030, with a CAGR of 10.8% between 2021 and 2030 (Dash and Suman, 2021).

The COVID-19 pandemic is anticipated to hinder the expansion of this market segment. Due to the strain on health systems around the world and the growing need for human treatment facilities and diagnostic technologies, investments in other areas were reduced (Carreiras et al, 2022).

We now constantly worry about potential conflict scenarios that play out on a European scale and which we may believe may have an impact on our businesses. Violent disputes show to have no influence on the establishment of new businesses, which does not seem to be proof for this (Brück et al, 2010).

The rise in demand for veterinary oncology therapy, the prevalence of animal cancer, the rise in funding from private and public organizations for animal care facilities, and the rise in research and development for oncology treatments for pets are the main drivers of the global veterinary oncology market's expansion (Dash and Suman, 2021).

Due to the development in R&D activities in the animal health sector, the rise in the number of

chemotherapy treatments in animals for the treatment of cancer, and the rise in the adoption of chemotherapeutic procedures in pets, chemotherapy dominated the market in 2020 and this trend is anticipated to continue. The number of research studies for the treatment of animals and the rise in the use of certain therapies for pets, however, are projected to cause the radiology segment to expand significantly (Figure 13) (Dash and Suman, 2021).

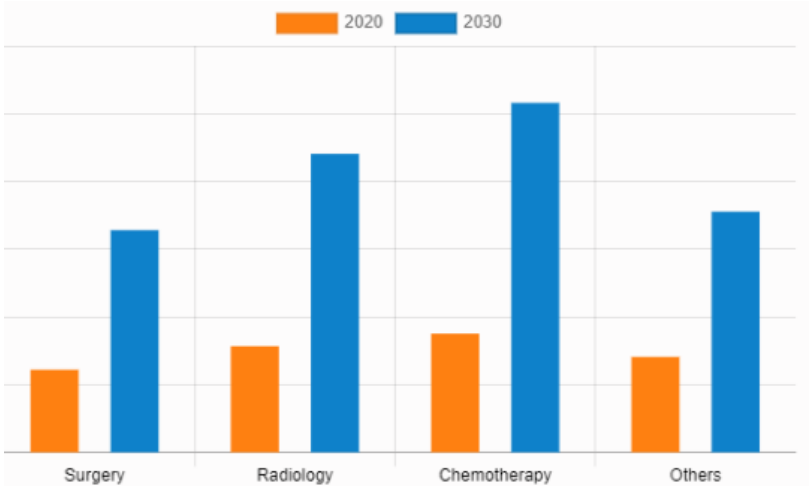

Figure 13: Segmented review of veterinary oncology market by therapy (Source: (Dash and Suman, 2021)).

The pharmaceutical market vet is about 30 times less than the worldwide pharmaceutical industry devoted to human health. Animal health is now the sole focus of business divisions of the majority of large pharmaceutical corporations. They only account for 5% of its overall profits (Capanema et al, 2007).

The market for animal health is expanding as a result of a shift in consumer behaviour, pet owners now view their animals as members of the family and place a higher priority on their welfare. The owners list food and health as their top concerns, and they also rank comfort and hygiene as major sources of worry (Appendix 3) (Capanema et al, 2007; Fragoso, 2022).

Few data are available at the veterinary level., some experts have estimated that there are close to 500 million dogs in the globe. According to pet food producers, there are 85 million individuals living in Europe (Overgaauw, 2020; Sykes et al., 2020). With an estimated 2.1 million canines living in Portuguese households, our nation's pet population is still behind that of the most industrialized European nations (Fragoso, 2022).

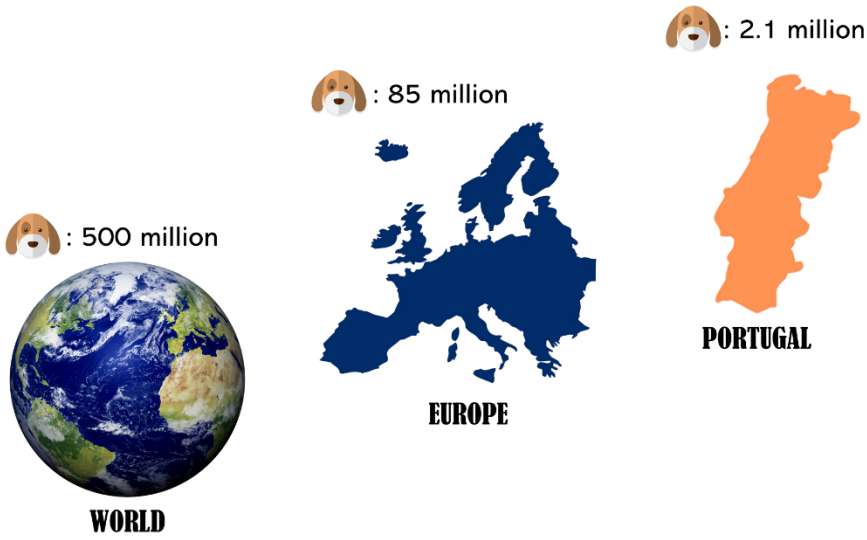

Figure 14: A visual depiction of the number of dogs in the world, in Europe and in Portugal (estimate).

Considering that male dogs are mostly unaffected by breast cancer, the majority of targets are female dogs. We may then narrow our attention to only half of the canine population by assuming that the percentage of the two sexes will be equal. One of the most common neoplasms among female dogs, who make up half of the canine population, is breast cancer, which affects 45% of them (Campos, 2020).

Table 5: Estimation of possible cases of breast cancer likely to be studied and of interest

	World	Europe	Portugal
Number of dogs (from data)	500 million	85 million	2.1 million
Female dogs (estimative)	250 million	42.5 million	1.05 million
Dogs with breast cancer (potential number)	112.5 million	19.125 million	473 thousand

With these figures in mind, we can thus predict that Portugal will have close to 473 thousand potential patients for our therapy. While there may be 19 million in Europe and 113 million worldwide (Table 5). After all the tests and regulations with the therapy of interest and when it is prepared for commercialization, the number of potential patients should represent a bigger number given the developing trend in the number of cancer cases that has been recorded over the course of so many years.

4.4 Competitors

A product's positioning, which is expressed by the relationship of position among competitors, is the image or identity that the product transmits to potential customers (Krejcar, 2020).The metal complex treatment is positioned as a solution for the treatment of breast cancer with easy reproducibility and more direct production, low systemic cytotoxicity, and a significant price advantage over some other treatments available right now (Appendix 2).

In an effort to address all the therapeutic limitations that the present treatments have

demonstrated, this treatment will be promoted as an alternative.

The biggest bottleneck that can then be pointed out to this treatment technology is its premature state with regard to tests, having only been carried out *in vitro* tests and still without even patenting, all results in live models are being measured. taking into account other compounds of the same family, then I intend this vanadium compound to have a similar behavior with regard to *in vivo* toxicity than the others and accumulation at the tumor site not in other organs such as liver and brain (Raposo et al., 2017; Rojas et al., 2022).

Bearing in mind that cancer treatment strategies have been showing a tendency to move towards targeted treatment strategies and if this is not the case, this can be seen as a disadvantage, however this will be a point that can be overcome with the subsequent functionalization. of the compost, if this proves to be necessary. When hospital waste is burned as part of the waste treatment process, chemotherapeutic compounds are released into the atmosphere. At this time, it is unknown whether or not these products will be harmful to us or our atmosphere/world. Given the condition of our planet and the responsibility we all have to preserve it, this can be noted as a potential drawback.

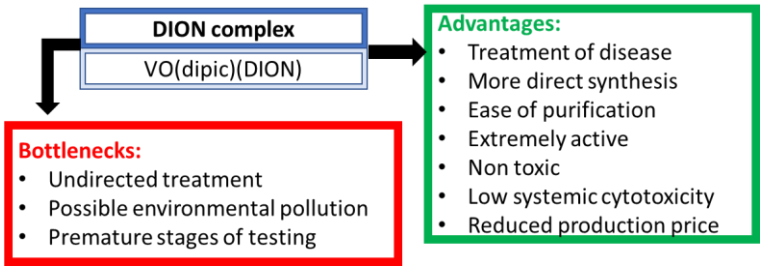

Figure 15: Illustrative diagram showing the benefits and drawbacks of DION complexes, namely the VO(dipic)(DION).

Medical experts in veterinary medicine that were consulted for this dissertation claim that the key elements of a successful treatment for these conditions are the treatment of the illness without endangering the rest of the animal's health because, in many cases, secondary conditions resulting from oncological treatment appear. The treatment itself, affect both healthy and tumor cells, which complicates cases where organ failure of vital organs for the animal's healthy life results, what is intended to be avoided through a suitor for new treatment. Animal carers really place the greatest emphasis on this; issues like expense and environmental pollution appear to be fairly minor concerns in their thinking (Appendix 1,3 and 4).

We can point to three main therapies competing with metal complexes mammary cancer treatment: surgery, chemotherapy and radiotherapy (Table 6).

Table 6: Main models of cancer treatment (surgery, chemotherapy and radiotherapy), their advantages and disadvantages

Surgery	Chemotherapy	Radiotherapy
-Visible tumor mass removals. -Most used method of treating breast cancer in dogs.	-Administration can be: oral, intravenous, intramuscular, subcutaneous, intrathecal and topical. -Acts on cells in process of division.	- Genetic damage to directly kill or induce apoptosis.
Advantages		
- Quick method.	- Prevention of tumor proliferation.	-Preservation of normal tissues and destruction of microscopic portions of neoplastic tissue. -No need for hospital admission.
Drawbacks		
- Limitation in the cure of metastases. - Evasive treatment. -Possible collateral damage: bleeding, blood clots, damage to nearby tissues/organs, drug reactions, pain, infection, slow recovery.	- There is no defined protocol for breast cancer in the bitch that has been shown to be effective.	-It does not demonstrate efficiency in the treatment of metastases. -Requires expensive equipment. -Not available in all countries.

Surgery is the most popular method of therapy for breast cancer in female dogs. Chemotherapy and radiation, the other two therapeutic options, have not yet demonstrated a generally effective regimen for treating the same condition and are frequently only employed as adjuvant therapy in this instance.

Despite being more commonly used, surgery also does not have the desired results, and the mortality associated with this type of cancer is still high. In addition, this technique is a very invasive modality, requiring hospitalization, and presents several possibilities of side effects. Breast cancer is also characterized by its high capacity to metastasize, with metastases occurring most of the deaths associated with breast cancer and not with primary tumors. Score. Point on which our treatment modality through the metallic DION complex would have the main advantage.

4.5 Business Model

The presentation of the central, cohesive structure of all foundational components through which a product/service generates value for its target customers is known as the business model (Krejcar, 2020). These has 3 essential components: value proposition, value capture and value delivery.

4.5.1. Value Proposition

How much value the idea creates and what advantages it will have when it is properly delivered is stated in the Value Proposition.

There have been few positive benefits in terms of lowering mortality over time, despite all the advancements achieved in the field of cancer, whether for humans or animals. Surgery continues to be

the preferred form of treatment for the cancer in study, insufficient to fight cancer most of the times, while other forms of treatment have essentially stagnated, there are no protocols for either chemotherapy or radiotherapy that have demonstrated significantly important results with regard to the effective cure of cancer.

The value proposition of the proposal of the treatment with the DION complex, VO(dipic)(DION) intended to be the delivery of a treatment that allows the effective fight against mammary cancer in dogs and does not negatively impact the welfare of the animal. As described previously in this chapter, these complexes are distinguished by having high activity and little systemic toxicity.

4.5.2. Value Capture

In the above section, the possibility of patentability was mentioned. The intellectual protection of the compound, not the treatment, would be our greatest weapon in capturing the value created, at the same time creating a barrier for competitors to take it. At the same point that the sale of the treatments to competent entities and patients would also allow to receive the value created in return.

In addition to the therapy's potential as a potent tool in the successful treatment of cancer, the patent will be our strongest tool for securing the complex at the market level and will also be our most effective means of generating profit.

By building long-lasting and reliable connections with the organizations to whom it delegated the distribution of treatment, we hope to expand sustainably and steadily increase the number of consumers.

The revenue model is predicated on the ongoing development of value and its capture, continuously placing bets on the high caliber of the generated product.

4.5.2.1. The power of veterinary health professionals in the dissemination of treatment

The power that advertising can have on the success or failure of a business cannot be ruled out.

Before talking about advertising, it is important to mention that in the veterinary environment, as well as in human medicine, there are prescription and non-prescription medicines (Fialho, 2018; Gomes, 2021).

Advertising of veterinary pharmaceutical products may then be directed to veterinarians and other animal health professionals, distributors and other entities legally authorized to transfer veterinary

medicinal products or holders' animals and the general public. Our treatment will be in the category of drugs subject to medical prescription. In other words, its advertising can no longer be addressed to the general public (animal holders included). Since our focus is all professionals in the area, it is important to establish a relationship of trust with them (Fialho, 2018; Gomes, 2021).. These will then be the ones who will or will not suggest our treatment as a treatment modality available to the target audience (Appendix 3).

4.5.3. Value Delivery

Value delivery is connected to the commercialization strategy, or how we plan to get our product to market, hopefully recouping the investment invested and compensating all the prior work put into treating the ailment being studied in animals. And it may involve the creation of a spin off from Nova University (or start-up).

With the intention of advancing the project, research and contacts were made in an attempt to proceed with *in vivo* tests, with no positive response in this regard, having to adapt the initial plan. So two value delivery strategies were introduced in our open, one of which would shift from patenting to patent sale to the pharmaceutical industry while the other would initially involve the patenting of the composite compound, setting aside the development, testing, and subsequent direct sale of the product. The figure below provides an overview of these two tactics (Figure 16).

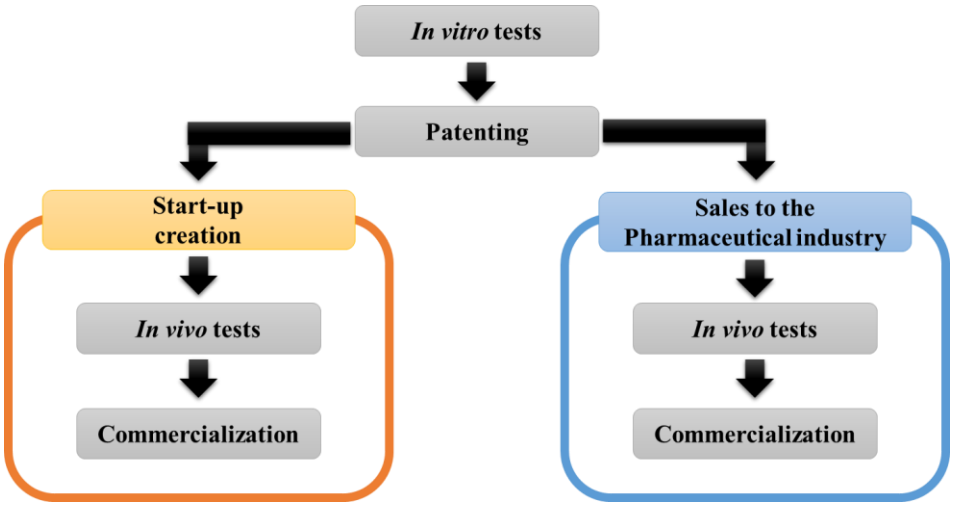

Figure 16: Diagram of the two business models: On the left is the model where, following patenting, a start-up is established that will be in charge of the subsequent in vivo testing and commercialization, and on the right is the model where, following the sale of the compound's patent to them, a pharmaceutical company will be in charge of these final two steps.

If a spin off from Nova University or start- up were to be created, we would have to make a bigger investment in both the formation of the start-up and workers and the *in vivo* testing that would be required before it could be sold. If we kept going in this direction, we would run into complications with the increasingly strict standards governing animal testing. The costs would be somewhat impracticable

for us, taking into account, for example, that for the performance of phase 1 *in vivo* tests only, in addition to the aforementioned regulation, the costs associated with them would be high, mainly due to the exams and follow-up analyses, in this case having two possible views, either these would be carried out by the owner, almost impossible to happen, or we would have to have access to outside investment or a grant to carry out this project.

Given that they will be able to move forward with the testing more readily, it appears that the method that entails patenting and then selling it to the pharmaceutical business is the most appropriate for this situation. Contacts in this direction have already been made, but they were unsuccessful due to the region's veterinary pharmaceutical institutes' lack of resources or their lack of interest as a result of the compounds' limited success and theoretically little worth since there was no patented.

4.6. Financial Projections: a preliminary overview

It is challenging to define a financial projection at this point of the research because it has just conducted *in vitro* testing and is still in its very early stages.

In addition to production expenses, the price of these medicines will be greatly influenced by the outcomes of *in vivo* examinations. *In vivo* experiments can also inform us the optimal way to administer the medication, such as orally or intravenously. As a result, these values will have a significant impact on the cost of therapy. They will also make it possible to estimate which doses will be required for treatment, without injuring the animal, and how many sessions of the same would be advised. It is challenging to define a financial model at this point of the research because it has just conducted *in vitro* testing and is still in its very early stages.

In this industry, the costs for newly introduced chemotherapy drugs are rising. The increased costs for new cancer therapies can be justified by the very specific targets of which one, therefore, only marketable to a small segment of patients. The higher prices allow the producers to recover the expense of development in the face of limited sales (Scalo,2014).

The price of new cancer innovations for the pharmaceutical industry is influenced by development costs, production costs, potential market size, competitors' prices, and the value of the new product on the market (Meropol et al., 2009).

For production costs what can you then make a small estimate of the cost of producing small portions of compost. However, this is always very relative, since there are certain variables that are complicated to estimate such as: electricity, water, equipment, liquid nitrogen, nitrogen line (gas), personal reagents for synthesis and analysis, such as nuclear magnetic resonance (Table 7).

Table 7: Costs of reagents to produce 15 grams of ruthenium compound with DION as ligand

Ruthenium precursor	240 €
Ligands	750+ 90 €
Solvent	271 €

The table shows the costs values of the reactants, in the scale-up of one of the tested ruthenium compounds, just to produce 15 grams of it. These data were provided by the researcher Oscar Rojas, involved in its synthesis. Having been told by the same that is always possible to optimize the reaction to obtain a greater amount, but this will also involve costs, but they can be adjusted if the reaction is done on a larger scale.

For the production 15 grams of the ruthenium complex cost, only in reagents, a total of 1351€. This will then serve for us as a small perspective of the order of how much it could cost to produce the compound from the family of compounds with DION as a ligand that will be commercialized, the vanadium complex, taking into account that the metallic center and most of the reactants could be different, with interference in the total cost (Appendix 2).

The final costs are not estimable and depend a lot on the scale of production, so far only small amounts of the complex have been synthesized when compared to what can be produced in an industrial environment. This large-scale production, in order to satisfy market needs, will lead to a significant cost reduction. At this moment, what we can assess is the price charged by the current competitors, radiotherapy was not taken into account as it is not a treatment modality practiced at national level, having the same as a reference for the definition of the possible price to be practiced. We then have where in Portugal, the price of the surgery is a fixed price that will be around 200/300 euros and the other possible modality of treatment, chemotherapy, costs are already less predictable, since each treatment session will cost around 200 euros, and the number of sessions will depend on the protocol and the case, which can cost well over 1000 euros. In addition to the costs associated with these treatments, we always have control analyses, such as blood collection and follow-up consultations, which can certainly add a cost of over 1000 euros to the entire process (Table 8) (Appendix 1).

Table 8: Summary of costs involved in the treatment of dog breast cancer in Portugal (surgery and chemotherapy)

	Surgery	**Chemotherapy**
Cost per session	200/300€	200€
Number of sessions	1	Protocol dependent
Adjacent costs	+1000€	

The metallic complex's suggested treatment is somehow similar to the present chemotherapy, thus it is reasonable to assume that the accompanying costs for the owner of the sick dog will be of a similar scale. Taking into account the fact that these chemicals' production was found to be more

straightforward and to be less expensive (Appendix 2) . In contrast, the object under investigation we are looking at offers a more compelling value proposition than any potential rivals, providing greater value that could support a higher price.

4.7. Final Remarks

Human genetics and cancer therapeutics at NOVA University of Lisbon is now testing metal center complexes with DION as a ligand. Throughout this endeavour, the goal is to in describing the marketing plan that will introduce our market with the highest success of this innovation. This makes it vital to define and clarify the laboratory's function throughout the marketing strategy's execution and in response to such inquiries as: Is it preferable to entrust production and marketing to a business already well-known in the health industry or to launch a start-up specifically for this purpose? Then it will is it important to have a distribution channel for the products when starting a start-up? Or is it acceptable to delegate this task to a third party due to the needs of the distribution market? These inquiries suggest a more thorough examination of the resources already available, the advantages and drawbacks of each solution, and the financial commitment necessary to complete the task.

In this chapter, a market analysis, business model and financial planning were carried out.

As for the market analysis, the veterinary oncology market is expected to have a growing trend over these years. Despite limited data about the veterinary world, an estimate was made and it is expected that of the 500 million dogs around the world approximately 112.5 million of them will develop breast cancer at some stage of their life, thus representing 112.5 million millions of potential customers.

This new technology intends to compete head-on with the technologies available so far for cancer treatment, surgery, chemo and radio (not available in Portuguese territory), this represents some advantages over these.

For the business model, among the two possibilities between creating a start-up or selling the patent to a pharmaceutical company, it was considered that in this case the second case will be more appropriate, due to all the necessary regulations and knowledge and training that would be needed to carry out tests on animals, resources that already exist in large industries, the creation of a start-up would make the whole process more expensive and delay the delivery of the product to the market. Due to the embryonic stage of the project, it is not yet possible to predict the cost that the treatment with the complex may have, being able only to observe the prices charged by others and analyze the cost of producing a reduced amount of compounds from the same family.

CHAPTER 5: CONCLUSIONS

In general, in terms of cancer treatments, despite all the advances that have been made and despite the increasing knowledge of the biological phenomena associated with the carcinogenesis process, there is still a high mortality associated with this disease. When it comes to breast cancer in the bitch, the treatment of choice is surgery, but this often proves to be insufficient, however, there are no other protocols, neither chemo nor radiotherapy that have been shown to be significantly effective.

During the present study, it was demonstrated that some of the compounds showed an effective antiproliferative potential against canine cancer cell lines, respectively manganese and vanadium. What was observed is that they present a low selectivity when compared with the results obtained in human fibroblasts, being even so the vanadium compound that presented a greater selectivity, although this comparison was being made with cells from different organisms, that is, different sensitivities.

Notably, data demonstrated by flow cytometry that possibly a non-apoptotic mechanism of cell death, autophagy, was mostly the programmed death process responsible for the loss of viability, with ROS contribution. With these compounds, there was also a decrease in the ability of wound healing and cell migration, with special relevance for the manganese compound, JHOR9, which showed a lower cell migration than when cells were exposed to the known drug, doxorubicin.

Of all the compounds tested so far, vanadium seems to be the most suitable to proceed to *in vivo* tests and then to the market, not only because of the results demonstrated in the laboratory, but also because it is one of the few that could be patented, which will be essential part of our business model.

When analysing the oncology market, it was found that this is an expanding market. With great investment potential, even more revealing the gaps in the capacity to deal with the treatment of cancers as common as the dog's breast cancer.

A business strategy and financial planning were difficult steps to define due to the prematurity of the project, and the next steps to follow are preponderant in its follow-up. What was found is that when it came to the business model, one could follow the creation of a start-up or opt for the path of selling the patent of the compound to the pharmaceutical industry, the second model seemed more suitable in the context of the project, due to to their better knowledge of the market and the regulation (extremely important), streamlining the entire process, being able to reduce costs for the customer and faster arrival of the technology to the same. In terms of the financial plan, the price to be charged is somehow related to the results that can be obtained in in vivo tests, not being able to make an estimate, being able to only observe for the production costs of small amounts of compost and for the prices charged by other competitors.

Being a work still in an embryonic state, there are many steps that can be taken after this work has already been carried out, proposals for the next work can be found in the following section.

5.1. Future Work

The next step would then be to carry out these tests, *in vivo*, and within the scope of this thesis, several veterinarians and companies in the veterinary industry were contacted for this purpose, not having obtained a positive opinion, mainly due to lack of resources and also due to little by little tests still carried out with the complexes.

In order to add value to the complex and gain credibility in the market to later be able to find partners and resources to advance with the technology, the first step will be the patenting of the complex.

Post-patenting or during the process of the same, it would then be intended to proceed with the *in vivo* tests, for these it was then suggested the sale of the patent / partnership with a company in the pharmaceutical industry that already has all the materials and knowledge for such tests. What can be verified during the tests is that the delivery of the chemotherapeutic drug is not as directed as expected, and it may have to find a way to overcome this barrier, for example, vectoring the complex, using nanotechnology techniques.

Bearing in mind the significant similarities between female and female breast cancer, ultimately, in case of a positive opinion in veterinary use, one could consider translating the drug to use in human oncology, which, if possible, our last major project objective.

REFERENCES

Abdelmegeed, S. M., & Mohammed, S. (2018). Canine mammary tumors as a model for human disease. Oncology letters, 15(6), 8195-8205.

Akram, M., Iqbal, M., Daniyal, M., & Khan, A. U. (2017). Awareness and current knowledge of breast cancer. Biological Research, 50, 33.

Alcarazo, M. (2014). α-Cationic Phosphines: Synthesis and Applications. Chemistry–A European Journal, 20(26), 7868-7877.

Andrade, F. H., Figueiroa, F. C., Bersano, P. R., Bissacot, D. Z., & Rocha, N. S. (2010). Malignant mammary tumor in female dogs: environmental contaminants. Diagnostic pathology, 5(1), 1-5.

Anjomshoa, M., Torkzadeh-Mahani, M., Shakeri, M., & Adeli-Sardou, M. (2016). The Zn (II) nanocomplex: sonochemical synthesis, characterization, DNA-and BSA-binding, cell imaging, and cytotoxicity against the human carcinoma cell lines. Journal of fluorescence, 26(3), 1007-1020

Ayuso, J. M., Park, K. Y., Virumbrales-Muñoz, M., & Beebe, D. J. (2021). Toward improved in vitro models of human cancer. APL bioengineering, 5(1), 010902

Benavente, M. A., Bianchi, C. P., & Aba, M. A. (2016). Canine mammary tumors: risk factors, prognosis and treatments.

Bergman, P. J. (2007). Canine oral melanoma. Clinical techniques in small animal practice, 22(2), 55-60.

Beaudu-Lange, C., Larrat, S., Lange, E., Lecoq, K., & Nguyen, F. (2021). Prevalence of reproductive disorders including mammary tumors and associated mortality in female dogs. Veterinary Sciences, 8(9), 184.

Borecka, P., Ratajczak-Wielgomas, K., Ciaputa, R., Kandefer-Gola, M., Janus, I., Piotrowska, A., ... & Nowak, M. (2020). Expression of periostin in cancer-associated fibroblasts in mammary cancer in female dogs. in vivo, 34(3), 1017-1026.

Brück, T., Llussá, F., & Tavares, J. (2010). Perceptions, expectations, and entrepreneurship: The role of extreme events.

Calucci, L., Pampaloni, G., Pinzino, C., and Prescimone, A. (2006). Transition metal derivatives of 1,10-phenanthroline-5,6-dione: controlled growth of coordination polynuclear derivatives. Inorg. Chim.

Acta 359, 3911–3920.

Canadas, A., França, M., Pereira, C., Vilaça, R., Vilhena, H., Tinoco, F., ... & Santos, M. (2019). Canine mammary tumors: comparison of classification and grading methods in a survival study. Veterinary pathology, 56(2), 208-219.

Capanema, L. X. D. L., Souza, J. O. B. D., Velasco, L. O. M. D., & Noguti, M. B. (2007). Panorama da indústria farmacêutica veterinária.

Carreiras, H., Malamud, A., Pinéu, D., Rodrigues, D., Gomes, J. M., Fernandes, J. P. T., ... & Rodrigues, T. (2020). A nova (des) ordem mundial: efeitos da pandemia. IDN Brief..

Calderazzo, F., Pampaloni, G., & Passarelli, V. (2002). 1, 10-Phenanthroline-5, 6-dione as a building block for the synthesis of homo-and heterometallic complexes. Inorganica Chimica Acta, 330(1), 136-142.

Campos, J. (2020). Câncer de mama em cadelas: o desafio de diagnosticar de forma precoce! Blog do Mundo Veterinário.

Candellone, A., Saettone, V., Badino, P., Girolami, F., Radice, E., Bergero, D., ... & Meineri, G. (2021). Management of Feline Hyperthyroidism and the Need to Prevent Oxidative Stress: What Can We Learn from Human Research?. Antioxidants, 10(9), 1496.

Cekanova, M., & Rathore, K. (2014). Animal models and therapeutic molecular targets of cancer: utility and limitations. Drug design, development and therapy, 8, 1911.

Claudel, M., Schwarte, J. V., & Fromm, K. M. (2020). New antimicrobial strategies based on metal complexes. Chemistry, 2(4), 849-899.

Clemente, M., Pérez-Alenza, M. D., & Peña, L. (2010). Metastasis of canine inflammatory versus non-inflammatory mammary tumours. Journal of Comparative Pathology, 143(2-3), 157-163.

Coelho, T. F. (2013). Relatório de Estágio realizado na Farmácia Outeiro do Linho e no Centro Hospitalar do Porto.

Conn, S. J., Hocking, B., Dayod, M., Xu, B., Athman, A., Henderson, S., ... & Gilliham, M. (2013). Protocol: optimising hydroponic growth systems for nutritional and physiological analysis of Arabidopsis thaliana and other plants. Plant methods, 9(1), 1-11.

DaMatta, R. A. (2010). Modelos animais na pesquisa biomédica. Scientia Medica, 20(3), 210-211.

Dash L., Suman O. (2021).Global Opportunity Analysis and Industry Forecast, 2021—2030 Allied Market Research, 257

Deb, J., Lakshman, T. R., Ghosh, I., Jana, S. S., & Paine, T. K. (2020). Mechanistic studies of in vitro anti-proliferative and anti-inflammatory activities of Zn(II)-NSAID complexes of 1,10-phenanthroline-5,6-dione in MDA-MB-231 cells. Dalton Transactions.

Deegan, C., Coyle, B., McCann, M., Devereux, M., & Egan, D. A. (2006). In vitro anti-tumour effect of 1,10-phenanthroline-5,6-dione (phendione), [Cu(phendione)3](ClO4)2·4H2O and [Ag(phendione)2]ClO4 using human epithelial cell lines. Chemico-Biological Interactions, 164(1-2), 115–125.

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., ... & Gore, A. C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocrine reviews, 30(4), 293-342.

Do Nascimento Silva, M., & Martins, W. K. (2020). Modulação da autofagia como estratégia terapêutica contra o câncer. *Arquivos Catarinenses de Medicina*, *49*(3), 125-143.

Dobson, J. M. (2013). Breed-predispositions to cancer in pedigree dogs. International Scholarly Research Notices, 2013.

Dospivova, D., Smerkova, K., Ryvolova, M., Hynek, D., Adam, V., Kopel, P., ... & Kizek, R. (2012). Catalytic electrochemical analysis of platinum in Pt-DNA adducts. Int. J. Electrochem. Sci, 7(4), 3072-3088.

Eccles, B. K., Copson, E., Maishman, T., Abraham, J. E., & Eccles, D. M. (2015). Understanding of BRCA VUS genetic results by breast cancer specialists. BMC cancer, 15(1), 1-9.

Eremina, J. A., Ermakova, E. A., Smirnova, K. S., Klyushova, L. S., Berezin, A. S., Sukhikh, T. S., ... & Lider, E. V. (2021). Cu (II), Co (II), Mn (II) complexes with 5-phenyltetrazole and polypyridyl ligands: Synthesis, characterization and evaluation of the cytotoxicity and antimicrobial activity. Polyhedron, 206, 115352

Fagundes, D. J., & Taha, M. O. (2004). Modelo animal de doença: critérios de escolha e espécies de animais de uso corrente. Acta Cirúrgica Brasileira, 19, 59-65.

Fernandes, A. R., Jesus, J., Martins, P., Figueiredo, S., Rosa, D., Martins, L. M., ... & Baptista, P. V. (2017). Multifunctional gold-nanoparticles: A nanovectorization tool for the targeted delivery of novel

chemotherapeutic agents. Journal of Controlled Release, 245, 52-61.

Fialho, P. C. P. M. (2018). Comunicação e informação da indústria farmacêutica em medicina veterinária (Doctoral dissertation, Universidade de Lisboa (Portugal)).

Fragoso, S., Anjos, C., Almeida, A., Manteca, X., & de Sousa, L. (2022). Dog bites in Portugal: Analysis of incidents, and consequences for both people and dogs. Journal of Veterinary Behavior, 54, 19-27.

Freitas, F., Lima, A., Aparecida, S., & Arçari, D. (2011). Nanotecnologia empregada no tratamento do câncer. Gestão em Foco, 4(1), 1-6.

Freitas, D. C., & Muniz, B. V. (2020). Aplicações da nanotecnologia em fármacos para o tratamento do câncer de mama. Revista Científica Eletrônica de Ciências aplicadas da FAIT, (2).

Galluzzi, L., Baehrecke, E. H., Ballabio, A., Boya, P., Bravo-San Pedro, J. M., Cecconi, F., ... & Kroemer, G. (2017). Molecular definitions of autophagy and related processes. The EMBO journal, 36(13), 1811-1836.

Galluzzi, L., Kepp, O., Trojel-Hansen, C., & Kroemer, G. (2012). Non-apoptotic functions of apoptosis-regulatory proteins. EMBO reports, 13(4), 322-330.

Garden, O. A., Volk, S. W., Mason, N. J., & Perry, J. A. (2018). Companion animals in comparative oncology: One Medicine in action. The Veterinary Journal, 240, 6-13.

Gomes, J. C. F. (2021). A medicina veterinária e a resistência aos antimicrobianos em contexto" one health": a proposta de uma ferramenta de apoio à prescrição de antimicrobianos de uso veterinário (Doctoral dissertation, Universidade de Lisboa, Faculdade de Medicina Veterinária).

Goss, C. A., & Abruna, H. D. (1985). Spectral, electrochemical and electrocatalytic properties of 1,10-phenanthroline-5,6-dione complexes of transition metals. Inorganic Chemistry, 24(25), 4263–4267.

Granato, M. Q., Gonçalves, D. D. S., Seabra, S. H., McCann, M., Devereux, M., Dos Santos, A. L. S., & Kneipp, L. F. (2017). 1, 10-Phenanthroline-5, 6-dione–based compounds are effective in disturbing crucial physiological events of Phialophora verrucosa. *Frontiers in microbiology*, *8*, 76.

Gray, M., Meehan, J., Martínez-Pérez, C., Kay, C., Turnbull, A. K., Morrison, L. R., ... & Argyle, D. (2020). Naturally-occurring canine mammary tumors as a translational model for human breast cancer. *Frontiers in oncology*, *10*, 617.

Grivicich, I., Regner, A., & da Rocha, A. B. (2007). Morte celular por apoptose. Revista brasileira de cancerologia, 53(3), 335-343.

Gu, F., & Lev, B. (2001). Markets in intangibles: Patent licensing. Available at SSRN 275948.

Guo, S., Liang, Y., Murphy, S. F., Huang, A., Shen, H., Kelly, D. F., ... & Sheng, Z. (2015). A rapid and high content assay that measures cyto-ID-stained autophagic compartments and estimates autophagy flux with potential clinical applications. Autophagy, 11(3), 560-572.

Habala, L., & Valentová, J. (2018). Metal complexes in medicine and pharmacy-the past and the present I. Ceska a Slovenska farmacie: casopis Ceske farmaceuticke spolecnosti a Slovenske farmaceuticke spolecnosti, 67(5-6), 182-191.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. cell, 144(5), 646-674.

Henry, C. M., Hollville, E., & Martin, S. J. (2013). Measuring apoptosis by microscopy and flow cytometry. Methods, 61(2), 90-97.

Hernandez, E., Llonch, P., & Turner, P. V. (2022). Applied animal ethics in industrial food animal production: exploring the role of the veterinarian. Animals, 12(6), 678.

Howe, L. M. (2015). Current perspectives on the optimal age to spay/castrate dogs and cats. Veterinary Medicine: Research and Reports, 6, 171.

Jan, R., Khan, M. S., Hassan, N., Mushtaq, U., Khanday, F. A., & Bhat, M. A. (2021). Synthesis, surface activity, self-aggregation and cytotoxicity of ruthenium (II) and Oxovanadium (IV) based metallo-surfactants. Journal of Molecular Liquids, 331, 115696

Kaplanis, M., Stamatakis, G., Papakonstantinou, V. D., Paravatou-Petsotas, M., Demopoulos, C. A., & Mitsopoulou, C. A. (2014). Re (I) tricarbonyl complex of 1, 10-phenanthroline-5, 6-dione: DNA binding, cytotoxicity, anti-inflammatory and anti-coagulant effects towards platelet activating factor. Journal of inorganic biochemistry, 135, 1-9.

Karges, J. (2020). Combining inorganic chemistry and biology: the underestimated potential of metal complexes in medicine. ChemBioChem, 21(21), 3044-3046.

Kaszak, I., Ruszczak, A., Kanafa, S., Kacprzak, K., Król, M., & Jurka, P. (2018). Current biomarkers of canine mammary tumors. Acta veterinaria scandinavica, 60(1), 1-13.

Kaur, G., Ramirez, A., Xie, C., Clark, D., Dong, C., Maki, C., ... & Hao, J. (2022). A double-blinded placebo-controlled evaluation of adipose-derived mesenchymal stem cells in treatment of canine atopic dermatitis. Veterinary Research Communications, 46(1), 251-260.

Kim, J., Koo, B. K., & Knoblich, J. A. (2020). Human organoids: model systems for human biology and medicine. Nature Reviews Molecular Cell Biology, 21(10), 571-584.

Klopfleisch, R., Von Euler, H., Sarli, G., Pinho, S. S., Gärtner, F., & Gruber, A. D. (2011). Molecular carcinogenesis of canine mammary tumors: news from an old disease. Veterinary pathology, 48(1), 98-116.

Kowalski, S., Wyrzykowski, D., & Inkielewicz-Stępniak, I. (2020). Molecular and cellular mechanisms of cytotoxic activity of vanadium compounds against cancer cells. Molecules, 25(7), 1757.

Krejcar, O., Frischer, R., Hlavica, R., Kuca, K., Maresova, P., & Selamat, A. (2020). Review of available SW solutions for intellectual property management systems from the perspective of open innovation. Journal of Open Innovation: Technology, Market, and Complexity, 6(2), 23.

Krishnaveni, V., & Kumaraguru, N. (2017). Studies on DNA Interaction, Biological Activities of Surfactant-Cobalt(III)-Phendione Complexes. Journal of Pharmaceutical and Medicinal Research. s. - Volume 3 Issue 1 68–72

Lages, L. F. (2016). VCW—Value Creation Wheel: Innovation, technology, business, and society. Journal of Business Research, 69(11), 4849-4855.

Lages, L. F., Ricard, A., Hemonnet-Goujot, A., & Guerin, A. M. (2020). Frameworks for innovation, collaboration, and change: Value creation wheel, design thinking, creative problem-solving, and lean. Strategic Change, 29(2), 195-213.

Lannagan, T. R., Jackstadt, R., Leedham, S. J., & Sansom, O. J. (2021). Advances in colon cancer research: in vitro and animal models. Current opinion in genetics & development, 66, 50-56.

LeBlanc, A. K., & Mazcko, C. N. (2020). Improving human cancer therapy through the evaluation of pet dogs. *Nature Reviews Cancer*, *20*(12), 727-742.

Lenis-Rojas, O. A., Carvalho, B., Cabral, R., Silva, M., Friães, S., Roma-Rodrigues, C., ... & Royo, B. (2022). Manganese (I) tricarbonyl complexes as potential anticancer agents. JBIC Journal of Biological Inorganic Chemistry, 27(1), 49-64.

Li, F., Guo, H., Yang, Y., Feng, M., Liu, B., Ren, X., & Zhou, H. (2019). Autophagy modulation in

bladder cancer development and treatment. Oncology reports, 42(5), 1647-1655.

Lieschke, G. J., & Currie, P. D. (2007). Animal models of human disease: zebrafish swim into view. Nature Reviews Genetics, 8(5), 353-367.

Loreto, C., La Rocca, G., Anzalone, R., Caltabiano, R., Vespasiani, G., Castorina, S., ... & Sansalone, S. (2014). The role of intrinsic pathway in apoptosis activation and progression in Peyronie's disease. BioMed research international, 2014.

Mak, I. W., Evaniew, N., & Ghert, M. (2014). Lost in translation: animal models and clinical trials in cancer treatment. American journal of translational research, 6(2), 114.

Marchi, S., Giorgi, C., Suski, J. M., Agnoletto, C., Bononi, A., Bonora, M., & Pinton, P. (2012). Mitochondria-ros crosstalk in the control of cell death and aging. Journal of signal transduction, 2012.

Martins, A. S., Marques, M. P. M., & Gil, A. M. (2016). 5. Metabolómica e complexos metálicos: aliados no combate ao cancro?. Bioquímica e Bem Estar@ UA 2016.

Mattiuzzi, C., & Lippi, G. (2019). Current cancer epidemiology. Journal of epidemiology and global health, 9(4), 217.

McCann, M., Coyle, B., Mckay, S., McCormack, P., Kavanagh, K., Devereux, M., et al. (2004). Synthesis and X-ray crystal structure of [Ag(phendio)2]ClO4(phendio = 1,10-phenanthroline-5,6-dione) and its effects on fungal and mammalian cells. Biometals 17, 635–645.

McCann, M., Santos, A. L. S., Silva, B. A., Romanos, M. T. V., Pyrrho, A. S., Devereux, M., et al. (2012). In vitro and in vivo studies into the biological activities of 1,10-phenanthroline, 1,10-phenanthroline-5, 6-dione and its copper(II) and silver(I) complexes. Toxicol. Res. 1, 47–54.

McKenzie, L. K., Sazanovich, I. V., Baggaley, E., Bonneau, M., Guerchais, V., Williams, J. G., ... & Bryant, H. E. (2017). Metal Complexes for Two-Photon Photodynamic Therapy: A Cyclometallated Iridium Complex Induces Two-Photon Photosensitization of Cancer Cells under Near-IR Light. Chemistry–A European Journal, 23(2), 234-238.

Mendes, N., Dias Carvalho, P., Martins, F., Mendonça, S., Malheiro, A. R., Ribeiro, A., ... & Velho, S. (2020). Animal models to study cancer and its microenvironment. Tumor Microenvironment, 389-401.

Meropol, N. J., Schrag, D., Smith, T. J., Mulvey, T. M., Langdon Jr, R. M., Blum, D., ... & Schnipper, L. E. (2009). American Society of Clinical Oncology guidance statement: the cost of cancer care. Journal of Clinical Oncology, 27(23), 3868-3874.

Millimouno, F. M., Dong, J., Yang, L., Li, J., & Li, X. (2014). Targeting apoptosis pathways in cancer and perspectives with natural compounds from mother nature. Cancer prevention research, 7(11), 1081-1107.

Neubig, R. R., Spedding, M., Kenakin, T., & Christopoulos, A. (2003). International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. Pharmacological reviews, 55(4), 597-606.

Ndagi, U., Mhlongo, N., & Soliman, M. E. (2017). Metal complexes in cancer therapy–an update from drug design perspective. Drug design, development and therapy, 11, 599.

Nieto, A., Perez-Alenza, M. D., Del Castillo, N., Tabanera, E., Castano, M., & Pena, L. (2003). BRCA1 expression in canine mammary dysplasias and tumours: relationship with prognostic variables. Journal of Comparative Pathology, 128(4), 260-268.

Novosad, C. A. (2003). Principles of treatment for mammary gland tumors. Clinical techniques in small animal practice, 18(2), 107-109.

Oun, R., Moussa, Y. E., & Wheate, N. J. (2018). The side effects of platinum-based chemotherapy drugs: a review for chemists. Dalton transactions, 47(19), 6645-6653.

Overgaauw, P. A., Vinke, C. M., van Hagen, M. A., & Lipman, L. J. (2020). A one health perspective on the human–companion animal relationship with emphasis on zoonotic aspects. International journal of environmental research and public health, 17(11), 3789.

Pandey, U. B., & Nichols, C. D. (2011). Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. Pharmacological reviews, 63(2), 411-436.

Pang, L. Y., & Argyle, D. J. (2009). Using naturally occurring tumours in dogs and cats to study telomerase and cancer stem cell biology. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1792(4), 380-391.

Pecorino, L. (2021). Molecular biology of cancer: mechanisms, targets, and therapeutics. Oxford university press.

Pedrosa, P., Corvo, M. L., Ferreira-Silva, M., Martins, P., Carvalheiro, M. C., Costa, P. M., ... & Fernandes, A. R. (2019). Targeting cancer resistance via multifunctional gold nanoparticles. International journal of molecular sciences, 20(21), 5510.

Pinho, S. S., Carvalho, S., Cabral, J., Reis, C. A., & Gärtner, F. (2012). Canine tumors: a spontaneous animal model of human carcinogenesis. Translational Research, 159(3), 165-172.

Pivetta, T., Trudu, F., Valletta, E., Isaia, F., Castellano, C., Demartin, F., et al. (2014). Novel copper(II) complexes as new promising antitumour agents. A crystal structure of [Cu(1,10-phenanthroline-5,6-dione)2(OH2)(OClO3)]ClO4. J. Inorg. Biochem. 141, 103–113.

Queiroga, F. L., Perez-Alenza, M. D., Silvan, G., Pena, L., Lopes, C., & Illera, J. C. (2005). Role of steroid hormones and prolactin in canine mammary cancer. The Journal of steroid biochemistry and molecular biology, 94(1-3), 181-187.

Rahmati, M., Ebrahim, S., Hashemi, S., Motamedi, M., & Moosavi, M. A. (2020). New insights on the role of autophagy in the pathogenesis and treatment of melanoma. Molecular biology reports, 47(11), 9021-9032.

Ranieri, G., Gadaleta, C. D., Patruno, R., Zizzo, N., Daidone, M. G., Hansson, M. G., ... & Ribatti, D. (2013). A model of study for human cancer: Spontaneous occurring tumors in dogs. Biological features and translation for new anticancer therapies. Critical reviews in oncology/hematology, 88(1), 187-197.

Ranjan, K., Surolia, A., & Pathak, C. (2012). Apoptotic potential of Fas-associated death domain on regulation of cell death regulatory protein cFLIP and death receptor mediated apoptosis in HEK 293T cells. Journal of cell communication and signaling, 6(3), 155-168.

Rao, N. A., Van Wolferen, M. E., Gracanin, A., Bhatti, S. F., Krol, M., Holstege, F. C., & Mol, J. A. (2009). Gene expression profiles of progestin-induced canine mammary hyperplasia and spontaneous mammary tumors. J Physiol Pharmacol, 60(Suppl 1), 73-84.

Raposo, L. R., Roma-Rodrigues, C., Faísca, P., Alves, M., Henriques, J., Carvalheiro, M. C., ... & Fernandes, A. R. (2017). Immortalization and characterization of a new canine mammary tumour cell line FR37-CMT. Veterinary and comparative oncology, 15(3), 952-967.

Reis, A. D., Dalmolin, S. P., & Dallegrave, E. (2017). Modelos animais para avaliação auditiva: revisão de literatura. Revista CEFAC, 19, 417-428.

Robertson, N., & Cronin, L. (2002). Metal bis-1, 2-dithiolene complexes in conducting or magnetic crystalline assemblies. Coordination Chemistry Reviews, 227(1), 93-127.

Rojas, Ó. A. L. (2017). Diseño y estudio de compuestos de rutenio con actividad biológica (Doctoral dissertation, Universidade da Coruña).

Roy, S., Hagen, K. D., Maheswari, P. U., Lutz, M., Spek, A. L., Reedijk, J., & van Wezel, G. P. (2008). Phenanthroline derivatives with improved selectivity as DNA-targeting anticancer or antimicrobial drugs. ChemMedChem: Chemistry Enabling Drug Discovery, 3(9), 1427-1434.

Saha, S., Panigrahi, D. P., Patil, S., & Bhutia, S. K. (2018). Autophagy in health and disease: A comprehensive review. Biomedicine & Pharmacotherapy, 104, 485-495.

Senga, S. S., & Grose, R. P. (2021). Hallmarks of cancer—the new testament. Open biology, 11(1), 200358.

Seyfried, T. N., & Shelton, L. M. (2010). Cancer as a metabolic disease. Nutrition & metabolism, 7(1), 1-22.

Scalo, J. F., & Rascati, K. L. (2014). Trends and issues in oncology costs. Expert review of pharmacoeconomics & outcomes research, 14(1), 35-44.

Selo, M. A., Sake, J. A., Kim, K. J., & Ehrhardt, C. (2021). In vitro and ex vivo models in inhalation biopharmaceutical research—advances, challenges and future perspectives. Advanced Drug Delivery Reviews, 177, 113862.

Singh, V. K., & Seed, T. M. (2021). How necessary are animal models for modern drug discovery?. Expert opinion on drug discovery, 16(12), 1391-1397.

Silva, T. F., Martins, L. M., da Silva, M. F. C. G., Fernandes, A. R., Silva, A., Borralho, P. M., ... & Pombeiro, A. J. (2012). Cobalt complexes bearing scorpionate ligands: synthesis, characterization, cytotoxicity and DNA cleavage. Dalton Transactions, 41(41), 12888-12897.

Silva, A., Luís, D., Santos, S., Silva, J., Mendo, A. S., Coito, L., ... & Fernandes, A. R. (2013). Biological characterization of the antiproliferative potential of Co (II) and Sn (IV) coordination compounds in human cancer cell lines: a comparative proteomic approach. Drug metabolism and drug interactions, 28(3), 167-176.

Silva, T. F., Smoleński, P., Martins, L. M., Guedes da Silva, M. F. C., Fernandes, A. R., Luis, D., ... & Pombeiro, A. J. (2013). Cobalt and Zinc Compounds Bearing 1, 10-Phenanthroline-5, 6-dione or 1, 3, 5-Triaza-7-phosphaadamantane Derivatives–Synthesis, Characterization, Cytotoxicity, and Cell Selectivity Studies. European Journal of Inorganic Chemistry, 2013(21), 3651-3658.

Sivandzade, F., Bhalerao, A., & Cucullo, L. (2019). Analysis of the mitochondrial membrane potential using the cationic JC-1 dye as a sensitive fluorescent probe. Bio-protocol, 9(1), e3128-e3128.

Sterckx, S. (2010). Is the Non-Patentability of “Essentially Biological Processes” Under Threat?. The Journal of World Intellectual Property, 13(1), 1-23.

Stockert, J. C., Horobin, R. W., Colombo, L. L., & Blázquez-Castro, A. (2018). Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. *Acta histochemica*, *120*(3), 159-167.

Stratmann, N., Failing, K., Richter, A., & Wehrend, A. (2008). Mammary tumor recurrence in bitches after regional mastectomy. Veterinary Surgery, 37(1), 82-86.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians, 71(3), 209-249.

Sykes, N., Beirne, P., Horowitz, A., Jones, I., Kalof, L., Karlsson, E., ... & Larson, G. (2020). Humanity’s best friend: a dog-centric approach to addressing global challenges. Animals, 10(3), 502.

Thrall, D. E., & Gillette, E. L. (1995, August). Soft-tissue sarcomas. In Seminars in veterinary medicine and surgery (small animal) (Vol. 10, No. 3, pp. 173-179).

Thorburn, A. (2008). Apoptosis and autophagy: regulatory connections between two supposedly different processes. *Apoptosis*, *13*(1), 1-9.

Timmermans-Sprang, E. P., Gracanin, A., & Mol, J. A. (2017). Molecular signaling of progesterone, growth hormone, Wnt, and HER in mammary glands of dogs, rodents, and humans: new treatment target identification. Frontiers in Veterinary Science, 4, 53.

Trepat, X., Chen, Z., & Jacobson, K. (2012). Cell migration. Comprehensive Physiology, 2(4), 2369.

TungáLeung, C. W., YipáLam, J. W., & ZhongáTang, B. (2014). A dual functional AEE fluorogen as a mitochondrial-specific bioprobe and an effective photosensitizer for photodynamic therapy. Chemical communications, 50(92), 14451-14454.

Üstün Alkan, F., Bakirel, T., Üstüner, O., & Yardibi, H. (2014). In vitro effects of doxorubicin and deracoxib on oxidative-stress-related parameters in canine mammary carcinoma cells. Acta Veterinaria Hungarica, 62(3), 372-385.

Valdivia, G., Alonso-Diez, Á., Pérez-Alenza, D., & Peña, L. (2021). From conventional to precision therapy in Canine Mammary Cancer: A comprehensive review. Frontiers in Veterinary Science, 8.

Varol, M., Benkli, K., Koparal, A. T., & Bostancıoğlu, R. B. (2018). Design and synthesis of novel organometallic complexes using boronated phenylalanine derivatives as potential anticancer agents. Drug and Chemical Toxicology, 1–8.

Vascellari, M., Capello, K., Carminato, A., Zanardello, C., Baioni, E., & Mutinelli, F. (2016). Incidence of mammary tumors in the canine population living in the Veneto region (Northeastern Italy): Risk factors and similarities to human breast cancer. Preventive Veterinary Medicine, 126, 183-189.

Viganor, L., Galdino, A. C., Nunes, A. P., Santos, K. R., Branquinha, M. H., Devereux, M., et al. (2016). Anti-Pseudomonas aeruginosa activity of 1,10-phenanthroline-based drugs against both planktonic- and biofilm-growing cells. J. Antimicrob. Chemother.

Wang, P., Henning, S. M., & Heber, D. (2010). Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. PloS one, 5(4), e10202.

Wang, X., Simpson, E. R., & Brown, K. A. (2015). Aromatase overexpression in dysfunctional adipose tissue links obesity to postmenopausal breast cancer. The Journal of steroid biochemistry and molecular biology, 153, 35-44.

Welch, D. R., & Hurst, D. R. (2019). Defining the hallmarks of metastasis. Cancer research, 79(12), 3011-3027.

Wu, Q., Maskus, M., Pariente, F., Tobalina, F., Fernández, V. M., Lorenzo, E., & Abruña, H. D. (1996). Electrocatalytic Oxidation of NADH at Glassy Carbon Electrodes Modified with Transition Metal Complexes Containing 1,10-Phenanthroline-5,6-dione Ligands. Analytical Chemistry, 68(20), 3688–3696.

Yadav, S., & Singh, V. P. (2021). Alternatives to Animal Experiments in Research and Regulatory Testing. In Essentials of Laboratory Animal Science: Principles and Practices (pp. 137-156). Springer, Singapore.

Yu, L., Chen, Y., & Tooze, S. A. (2018). Autophagy pathway: cellular and molecular mechanisms. Autophagy, 14(2), 207-215.

Zatloukal, J., Lorenzova, J., Tichý, F., Nečas, A., Kecova, H., & Kohout, P. (2005). Breed and age as risk factors for canine mammary tumours. Acta Veterinaria Brno, 74(1), 103-109.

APPENDICES

Appendix 1: Summary table of the interview with the veterinary doctor, Rita Pinho Lopes (January 11, 2022)

SKILLS	
Specialty	Small animal clinic. Surgery for dogs and cats.
Years of involvement in veterinary practice	Almost 23 years ago.
Link to university/research in the veterinary field	No.
Use of innovative/little tried treatments	They're not really accessible. Therefore, little tried drugs are not innovative, some do appear from time to time. One or another that interests us we have already tried, but in fact we don't get to the clinic, clinical practice, things that are little tried, it's not very easy.
Work with clinics abroad	Not in partnership, he was in some places outside, even more when he was finishing the course, as an internship, but in partnership he did not work with any clinic.
INCIDENCE, MORTALITY AND FUTURE TRENDS OF CANINE CANCER	
Types of cancers that appear most frequently in veterinary clinics	In dogs, perhaps the most frequent are breast tumors, they are very common in dogs and cats, in fact, in female dogs they are very common. It is also very common for melanomas, carcinomas, fibrosarcomas, lipomas to appear, which are even benign tumors, but they do appear and maybe lymphomas.
Ease of diagnosis	On the one hand comes practice and clinical experience, but on the other hand we have laboratories that are working very well. There are tumors that are more frequent in a certain breed or in a certain age group or have a location in the animal that already tells us more or less what it can be, the appearance and thus, this concerns clinical practice. Then I could confirm, as in human medicine, with biopsies, and there are laboratories working very well in this area here in Portugal, so samples are taken, sent to the laboratory and always being able to know for sure what is going on there.
Cancer with the highest mortality	Not being absolutely sure, pointed to lymphoma. This can appear in various organs at various stages of evolution, sometimes not diagnosed so early. And as they can appear in various organs, whether in dogs or cats, sometimes they are inoperable and sometimes those that are inoperable we do chemotherapy treatments, but maybe they are the ones that lead to more deaths.
Canine mammary cancer	When you get breast cancer they have a resolution. They are very common in dogs and cats, but the resolution is not that complicated, it involves surgery and we are often successful in this surgery, when it has not progressed much and we do not have metastases.
Observation of a growing trend in the number of cancer cases	Yes, in all, it probably has to do with cancers in animals, it is often not possible to know the cause, but it has genetic, environmental, nutritional, hormonal causes and, therefore, yes, like people, there has been an increase in frequency. It is also no less true that people are more aware, they appear more at the clinic.
Similarity of canine and human cancers	Yes, in some cases yes.
EXISTING TYPES OF TREATMENT	
Most used canine cancer treatments	The first approach whenever possible is surgical. Many cancers whose solution is surgical and until a few years ago this was the only thing that could be done. Currently, chemotherapeutic treatments are much more widespread, not being very similar to those of humans, we already have a range of chemotherapeutic treatments that are used in canine treatments. Sometimes owners can't move forward because they have no economic possibility to pay for treatments that are really very expensive, but that there is already a lot on the market, and the first approach in most of them, at least in my opinion it is surgical.
Prices	Surgery is a treatment that begins and ends, the person has a value of 200-500 euros. The problem is chemotherapy treatments, people spend around 200 euros per treatment and the treatment is weekly. Then it depends on the protocols, but the complete treatment far exceeds one thousand euros. It's not just the treatment, but everything else that is done as in human medicine, doing control of analyses, taking blood frequently to see how the animal is evolving, follow-up consultations, that is, everything together is surely more than 1000 euros in a simple protocol, because it can evolve much more than that.

Variables to consider when choosing the most appropriate treatment	First comes the type of cancer and for us veterinarians, along with the suffering of the animal, then the cost of treatment has to come without a doubt, as I told you, it is an extremely important factor. And then finally the characteristics of the animal.
EXPECTATIONS REGARDING A NEW TREATMENT METHOD	
Test proposal of a new chemotherapeutic treatment	Of course, regulation. The promise of effectiveness or the expectation of effectiveness and animal suffering, which is a very decisive factor here. On the other hand, price and cost play a key role.
RESPONSIBILITY AND SOCIAL SCREENING	
Pollution by compounds and possible barrier to treatment	Handling veterinary products can be dangerous for us, so much so that there are products that are only handled by veterinarians and not by owners. It is important to be approved, in addition to animal safety, you have to take into account human safety Sustainability and the environmental footprint, nowadays you have to be careful with that.

Appendix 2: Summary table of the interview with the researcher, Oscar Rojas (March 8, 2022)

Is there reproducibility in the synthesis of compounds?	• In the vast majority of compounds, yes. • Most of the time, the reaction is performed 1 or 2 times to ensure the reproducibility of the reaction
Could there be any difficulties for the large-scale production of compounds? Scale-up may not be possible?	• The more steps the reaction has, the more complicated the scale-up will be. • Basically, doing large-scale production is different from doing small-scale production, as the yield will be different than usual. • Sometimes it is better to do several small reactions than to do just one large reaction.
What factors can lead to a non-possibility of scale-up?	• The number of steps involved in the synthesis.
Since the main source of costing a treatment with the compounds will be associated with the cost of the synthesis process, can you estimate prices for it?	• It is difficult to predict, as it varies depending on the metallic compound. • We have elements such as ruthenium which are more expensive, then we have elements such as manganese which are more abundant, that is, it is cheaper. • The ligand will also influence the price. • There are treatments currently used that are much more expensive than the use of metal complexes.
What could make the synthesis process more expensive?	• A synthesis that requires a lot of heating. • The purification process is very time consuming and expensive. • The more steps, the more time and the more difficult the purification.
How long does the synthesis of a new compound take on average?	• Depends on the complex in question, generally between 2 to 4 days.
What do you think is the biggest advantage of using these complexes as a treatment for cancer?	• More direct synthesis. • Purification is affordable. • DION complexes show great activity. • They do not show in vitro and in vivo toxicity.
Do you recognize the potential of these DION compounds in in vivo treatments?	•Yes, a lot. These are very active and non-toxic, unlike platinum compounds that have this problem.
Could the use of these complexes as treatment have an impact on the health of animals/people or even on the environment?	• At the environmental level, it is difficult to predict, because if applied in clinical practice, their waste will be treated in a specific way that it is not known if it could lead to problems. • As for health in living beings, no toxicity was observed with this family of compounds in zebrafish embryos, even using high concentrations of the compounds (500 mg), so it is expected that there will be no problems for higher living beings. • Xenografts were also used in the in vivo toxicity study and compounds from this family decreased the number of cancer cells without inducing toxicity

Appendix 3: Summary table of the interview with the veterinary doctor, Ana Eira (March 11, 2022).

SKILLS	
Specialty	It does not have any specialty recognized by the European or American college. But he has an area of interest that is oncology and abdominal ultrasound.

Years of involvement in veterinary practice	Since 2006, that is, 16 years.
Link to university/research in the veterinary field	No.
Use of innovative/little tried treatments	The treatments they administer are always something experienced, generally in other countries. For them to test new treatments is not clinical practice. The most common treatment used in the clinic today is electrochemotherapy.
Work with clinics abroad	She has already visited some such as the Royal College and University of Tennessee.
INCIDENCE, MORTALITY AND FUTURE TRENDS OF CANINE CANCER	
Types of cancers that appear most frequently in veterinary clinics	Lymphoma, whether in dogs or cats. Then in dogs we have skin tumors and mast cell tumors, angiosarcoma, osteosarcoma and breast cancer. And breast cancer and skin fibrosarcomas in cats.
Ease of diagnosis	It really depends on the tumor. Most are easily diagnosed and identifiable by owners and at a diagnostic appointment. Others are more complicated, especially those that arise at the level of internal organs.
Cancer with the highest mortality	Melanomas are very difficult to treat, often the diagnosis is made in very advanced stages of cancer with a high level of metastases.
Canine mammary cancer	70% are benign and 30% are malignant, of which ¼ are treatable with surgery alone. The remaining grade 2 and 3 often metastasize to the lymph node and lung, which leads to the death of dogs due to metastasis.
Observation of a growing trend in the number of cancer cases	Yes, a lot. Factors: - Animals live longer - Environmental factors (food/pollution). City animals end up having higher rates of cancer, but they also live longer than others, in rural environments, which usually die earlier due to other diseases, such as leishmaniasis. - Trend related to races (genetics) - Greater investment by people with regard to the health of their animals. -More accurate diagnoses, knowledge of the causes of death of dogs.
Similarity of canine and human cancers	There are many and there are already several studies about it. There are comparative oncology organizations.
EXISTING TYPES OF TREATMENT	
Most used canine cancer treatments	In Portugal, after surgery, the only possible option is chemotherapy, which is even the main treatment for inoperable cancers. In Portugal, there is no institution with conditions to offer radiotherapy, it is inconceivable for any clinic in Portugal to make investments in this regard, given the price of the material and its maintenance. For those who are able to do so, they can only access this treatment if they are transferred to other countries, such as Spain or France. The greatest hope at this moment is that with the support of the state at the level of academies there will be adaptation of some machine in disuse in human medicine in veterinary practice. In addition to conventional chemotherapy, what is most different at the moment offered by the clinic is electrochemotherapy.
Most commonly used chemotherapeutic treatments	Vincristine + Cyclophosphamide + Doxorubicin (treatment of lymphomas) Lomustine Cisplatin (highly toxic in cats, very toxic in handling) Vinblastine Tocenarib
Efficacy of chemotherapy treatments	Depends on the tumors. In the case of lymphomas, for example, these lead to a long period of remission of the disease, which is very positive. Chemotherapy is often used for palliative purposes, to improve living conditions and not to treat the disease itself, with the aim of delaying the progression of the disease. Most animals benefit from treatment, whether it is remission, cure or simply improvement of living conditions. Chemotherapy is not a miracle cure.
Disadvantages of chemotherapy	Care must be taken with pets in treatment that live with small children. Costs, many are not able to afford the costs of treatments. Treatments can induce side effects (often overcome by other treatments).
Variables to consider when choosing the most appropriate treatment	Adapt according to the type of tumor. Greater or lesser possibility of people in terms of economics and time. Check what is most beneficial for the animal.
EXPECTATIONS REGARDING A NEW TREATMENT METHOD	

Test proposal of a new chemotherapeutic treatment	It would be difficult, there is no such openness at the hospital and client level. University institutions may be more open to this type of test. It causes some distress not to know the effect of these treatments tested practically only in vitro in dogs. But it doesn't completely turn its back on this type of approach, but the molecule has to be very well grounded. Clients of the clinic, when they arrive there, go looking for more experienced information, they do not see much openness on their part.
RESPONSIBILITY AND SOCIAL SCREENING	
Pollution by compounds and possible barrier to treatment	The main objective in the treatment is to treat the dogs, the possible impacts that can arise from them are always a concern, but they are not the main focus. There are always some precautions to be taken, the use of the molecule is not abused just because and the hospital is very careful with regard to the way in which these residues are dispensed. As for the care that people take during these treatments, it is more complicated to control, many recommendations are given in order to reduce environmental pollution, such as disinfecting with bleach, but many people neglect this process. More than fear of the impacts they may have on the environment, they are more concerned about the impacts they may have on other beings. For example, in the case of an intravenous treatment, there is a great excretion in the following 24/48 hours of residues and since the children are in a stage of development of their immune system, it is necessary to avoid their contact with animals in treatment.

Appendix 4: Summary table of the interview with doctors, Alexandre Trindade e Maria João Soares (March 30, 2022)

Needs to advance dog studies:	• Obtain consents from DGAV and the ethics committee. • Make an alignment of the experience (know which controls, ...).
Critical points of this process:	• Need to secure funding. • Great complexity of the dossier to be delivered to the competent authorities. • Obtained and significant results.
Financing need:	With no funding: • Costs would have to be borne by the animal owners (less acceptance of project participation) • Some clinics participating in the project would even be able to afford the costs of some necessary follow-up tests, but not all (ultrasound, blood count, radiography, ...)
Possible experience design:	• Need to get funding • Regulatory part (lengthy process) • First, before getting to phase 2 tests, trying out the complexes in bitches with cancer, it would be necessary to do phase tests on healthy dogs. • Knowledge of patient recruitment processes. • It would have to be done in a multicentric context (a single veterinary center will not have the necessary samples). • Test on patients without any kind of alternative.

Appendix 5: Summary table of the interview with doctor, Diogo Pereira (June 28, 2022)

• Attempt of new contacts in the veterinary pharmaceutical industry.

Appendix 6: Summary table of the interview with doctor, Joaquim Henriques (July 13, 2022)

VETERINARY ONCOLOGY	
Current scenario:	• Veterinary oncology has been the subject of extensive investigation. • It involves many billions of euros. • Diagnostics have advanced. • They serve as a comparative study for human oncology. • There is a lot of market where to sell the product. • After validation in the animal, it is usually transferred to a human model.
Canine breast cancer:	• Very heterogeneous, as of the woman. • One of the most interesting to be studied, high incidence in both bitches and women. • Very interesting to study the phenotype and see if there are mutations or not. • Treatment generally the same as humans. • Through the literature, we can see that we have more and more knowledge about carcinoma, but the treatment remains the same, with a great need for development.
IN VITRO TESTS:	
Results already obtained with our complexes:	• Interesting results for carcinoma. • Complexes do not accumulate in the liver or brain. • Little genetic cytotoxicity. • Improvements can be made, such as incorporating them into a liposome (not appearing to be necessary yet)
Competitors:	• Too early to talk about competitors.
IN VIVO TESTS:	
Clinical trials:	• Mouse models are often chosen as the translational model (low translation capacity) • Validation depends on the industry that purchased the patent. • There is a lot of product currently in the pipeline.
Costs:	• Costs nowadays do not prove to be a barrier in the development of a new treatment, the most important variables are toxicity and efficacy. • Putting the values in the treatment of a patient to give him another 3/4 months of life is around 6000 euros per day. • Few existing insurances.
Needs for clinical trials:	• Arrange sponsorship or submit a project in order to obtain financial funds. • A tutor will not want to pay for a trial treatment. • Have to withstand a lot of tests. • Unguarded animals cannot be tested (legislation). • Laboratory animals can be used. • Regulation (DGAV). • Contact Infarmed.
Timeline:	• It can take up to 1 year for a follow-up. • 1/3 month for acute cytotoxicity study. • It will always be necessary to know what happened to the animal in the long term after undergoing treatment. • All phase 1 tests (to study toxicity vs. therapeutic effect) have to be done before moving on to phase 2 tests.

Appendix 7: Metal complexes with DION as ligand on different cell lines

Metal	Formula	IC50 values (µM)		Notes	Reference
Ag (I)	$[Ag(DION)_2]ClO_4$	A-498	1.4	- Capable of decreasing cancer cell viability through an inhibition of DNA synthesis. - No involvement of either intercalation or mutation. -Don't cause an increased risk of genotoxicity.	(Deegan et al., 2006)
		Hep-G2	0.86		
		CHANG	0.3		
		HK-2	0.8		

Au (III)	Au(BPA)(DION)	A549	17.86	- Disruption of cytoskeleton integrity - BPA have the ability to transport itself across the cell membrane through the L-amino-acid-transport system - Lack of selectivity for cancer cells	(Varol et al., 2018)
		HUVECS	7.82		
Co (III)	$[Co(DION)_2(DA)Cl](ClO_4)_2$	MCF-7	16.3	- Can intercalate into DNA base pairs	(Krishnaveni and Kumaraguru, 2017)
	$[Co(DION)_2(DA)_2](ClO_4)_3$		12.2		
	$[CoCl(H_2O)(DION)_2][BF_4]$	HCT116	0.206	- Higher citoxicity than cisplatin and doxorubicin - Slight specificity to human colorectal carcinoma cell lines. -Vectorization platforms for these compounds can increase their efficacy - Activation of the expression of apoptotic genes - Cleaving agent in a concentration-dependent fashion by redox chemistry -Specific interaction with HAS - Decreased expression of anti-apoptotic proteins	(Silva et al., 2013)
		Hep-G2	0.582		
		MCF-7	0.69		
		FR37-CMT	1.39		(Raposo et al., 2017)
		MCF10A	5.14		
		Human Fibroblasts	0.611		(Silva et al., 2013)
Cu (II)	$[Cu(DION)_3](ClO_4)_2 \cdot 4H_2O$	A-498	0.88	- Capable of decreasing cancer cell viability through an inhibition of DNA synthesis - No involvement of either intercalation or mutation -Don't cause an increased risk of genotoxicity	(Deegan et al., 2006)
		Hep-G2	0.78		
		CHANG	0.2		
		HK-2	0.5		
	$[Cu(DION)_2(OH_2)(OClO_3)](ClO_4)$	CRL-7065	6.30	- High DNA binding constants -Appear to act via a different mechanism from that of cisplatin	(Pivetta et al., 2014)
		DU-145	4.10		
		HEP-G2	1.70		
		SK-MES-1	3.10		
		CCRF-CEM	3.80		
		CCRF-SB	1.21		
	$[Cu(DION)(PNT)_2]$	Hep2	1.7	- Higher cytotoxicity than cisplatin	(Eremina et al., 2021)
		MCF-7	0.96		
		Hep-G2	0.24		
Pt (III)	Pt(BPA)(DION)	A549	14.04	- Disruption of cytoskeleton integrity - BPA have the hability to transport itself across the cell membrane through the L-amino-acid-transport system - Lack of selectivity for cancer cells	(Varol et al., 2018)
		HUVECS	9.74		
	$Pt(DION)Cl_2$	A-498	4.9	- Conformational change in DNA - Similar to or more effective than cisplatin against five of the nine cell lines tested, including the cisplatin resistant line A2780R - Nuclease activity of complex was significantly lower than that of DION itself	(Roy et al., 2008)
		EVSA-T	3.05		
		H226	3.27		
		IGROV-1	2.67		
		M19-MEL	0.812		
		MCF-7	3.38		
		WIDr	19.5		
		A2780	0.827		
		A2780R	0.683		
Re (I)	$[Re(CO)_3(DION)Cl]$	T98G	> 50		(Kaplanis et al., 2014)
		PC-3	> 50		

		MCF-7	> 50	- Binds with the grooves of the DNA double helix retaining its overall structure - Cytotoxicity against all cell lines tested is almost completely lost	
Ru (II)	$[Ru(DION)_2(LPYA)]Cl_2$	HeLa	57	-Introduction of the amphiphilic ligand LPYA into their coordination sphere significantly increases the toxicity of these metal complexes.	(Jan et al., 2021)
V(IV)	$[VO(DION)(LPYA)]SO_4$	HeLa	57	-Introduction of the amphiphilic ligand LPYA into their coordination sphere significantly increases the toxicity of these metal complexes	(Jan et al., 2021)
		HEK-293	98		
Zn (II)	$[Zn(DION)(NPR)_2(H_2O)_2]$	RAW 264.7	2	- Inhibit cyclooxygenase pathway displaying anti-inflammatory activity	(Deb et al., 2020)
		MDA-MB-231	0.5		
	$[Zn(DION)(MFN)_2]$	RAW 264.7	1.7	-Inhibit cyclooxygenase pathway displaying anti-inflammatory activity -Delays in vitro cellular migration and down regulates EMT-related genes.	(Deb et al., 2020)
		MDA-MB-231	0.4		
	$[Zn(DION)_3](PF_6)_2]$	A549	55.7	- Complex binds to DNA electrostatic and via partial intercalative binding	(Anjomshoa et al., 2016)
		MCF–7	0.21		
	$[Zn(DION)_2]Cl_2$	FR37-CMT	1.05	-Vectorization platforms for these compounds can increase their efficacy -Reduction of viability was accompanied with a slight impairment of cell mobility	(Raposo et al., 2017)
		HCT116	0.217	- Higher citoxicity than cisplatin and doxorubicin.	(Silva et al., 2013)
		Hep-G2	0.978		
		MCF-7	0.73		
		Human Fibroblasts	0.60		
	$[ZnCl(\kappa O\text{-}PTA{=}O)(DION)][BF_4]$	HCT116	0.37		(Silva et al., 2013)
		Hep-G2	2.45		
		MCF-7	1.68		
		Human Fibroblasts	Between 1 and 2		