THESIS

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Effects of Chlorambucil and Firocoxib on Mammary Gland Cancer Cells

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Abstract

The thesis is concerned with the utilization of Chlorambucil and Firocoxib with regards to their effects and utilization for cancer treatment in the search to find chemo-therapeutic agents of a less harmful variety. A general review of the history and background of each drug is given to develop an understand of the roots of each of the chemical agents, followed by an in depth look at several studies carried out throughout the years before and after their release into the commercial market. Special care was also taken to review the affects when applied metronomically, a method of treatment which employs small consistent dosages over a long period of time as opposed to large less consistent dosing which has been shown to reduce the potentially harmful side effects that would otherwise be present from the utilization of these chemical agents. Finally, findings are summarized, and recommendation made for future study and analysis.

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List Of Abbreviations

Abbreviation	Meaning
ACE	angiotensin-converting enzyme
ALT	alanine aminotransferase test
AST	aspartate aminotransferase level
BALB/c	Bagg Albino lab bred mice
BAX	BCL2 associated X, apoptosis regulator
B-Cell	bursa of fabricus
Bcl-2	B-cell leukaemia/lymphoma 2 protein
BSAVA	British Small Animals Veterinary Association
CBC	complete blood count
CCT	cytological tumour classification
CHT	histological classification of the tumour
COX inhibitor	cyclooxygenase inhibitor
CRE	creatinine
DNA	deoxyribonucleic acid
DVM	Doctor of Veterinary Medicine
EMA	European Medicines Agency
eMC	electronic mmedicines ccompendium
FDA	Food and Drug Administration
GLP	good laboratory practice
GmbH	Gesellschaft mit beschränkter Haftung (Limited Liability Company)
IARC	International Agency for Research on Cancer
LSAB	labelled Streptavidin–Biotin
MPH	Master of Public Health
NN	number of nodules
NSAIDs	nonsteroidal anti-inflammatory drugs
OSU	Ohio State University
PGI-2	prostaglandin I2
pH	potential of hydrogen
P-value	statistical test of significance
SD	standard deviation
T-lymphocytes	thymus lymphocytes
UR	serum levels of urea
VMN	volume parameters of largest nodules
WHO	World Health Organization

1.0 Introduction

The following thesis is involved in the topic concerning mammary gland cancer and the pharmacological agents of chlorambucil and firocoxib and their effects which may be utilized to negate and treat the clinical and pathological detriments of mammary gland cancer.

The thesis will draw from several studies and peer reviewed projects to provide a wide variety of data and conclusive information on the on the success and methods of proper utilization of chlorambucil and firocoxib in a clinical setting for the treatment of mammary gland cancer. Furthermore, it aims to provide insight in possible future utilization. Attention will be given to the various side effects, their potential complications and the advised methods for avoidance in order to attain the most optimal utilization for the pharmacological agents at hand. Both Agents will be studied and reviewed separately as well as synergistically to properly establish a complete idea of their effects. The study will also be conducted from the aspects of the different types of mammary gland cancers such sarcomas and carcinomas as well as more benign forms and the efficacy of chlorambucil and firocoxib on each type determined. A final summary will be drawn up at the end to conclude on all the data gathered.

Mammary gland cancer is more prevalent in carnivorous animals, particularly those which have not undergone a spaying procedure (OSU Veterinary Medical Center, 2021). While mammary gland cancer is most certainly present in ruminant, swine and equine species; its prevalence is dogs and pronounced in these species, having only a reported presence of 1%-2% of neoplasia in slaughtered horses as reported by the Canadian Veterinary Medical Association (Boyce, S.D. , Goodwin, S.L, 2017), than in dogs and cats. Therefore the study will focus primarily on the aspects of treatment involving dogs, and will draw from references with regards to other species only when necessary to provide some unique insight on the potential effects of chlorambucil and firocoxib.

Mammary gland cancers are amongst the most common in canine species with a reported 50% of them developing into malignant forms as reported by the Ohio State University (OSU

Veterinary Medical Center, 2021), whereas in feline species mammary gland cancer accounts for 10% of all feline diagnosed tumours. Due to these statistics, it may be concluded that treatment of mammary gland cancer is of a primary importance in small animal medicine as it may be encountered quite frequently. While many surgical options for treatment are available and the surgical prevention method by spaying has substantial effects on ceasing the development of mammary gland cancer, these options are not always possible (Sorenmo, Shofer and Goldschmidt, 2008).

For example, spaying in breeding animals are not always the optimal choice as some of the surgical methods may be quite invasive and lead to a development of secondary complications. Therefore, the proper understanding and ability to utilize pharmacological agents such as chlorambucil and Firocoxib to treat mammary gland cancer on a clinical level to prevent the requirement for surgical intervention are of importance from both aspects concerning the health of the animal and the economic situations which may develop.

2.0 Chlorambucil

2.1 History and Introduction

Chlorambucil is a pharmacological agent with the chemical formula C14H19Cl2NO2 and contains a molar mass of 304.21g mol-1. Chlorambucil may be described as an alkylating agent of the nitrogen mustard type (Drug Information Portal - U.S. National Library of Medicine, 2021). Since is belongs to this group its history goes as far back as World War I, in which the utilization of mustard gas also known as sulphur mustard (C4H8CL2S) for chemical warfare was observed to show the pathological effects of a decreased leukocyte count. The first observation of these effects was performed by Dr. Edward Krumbhaar in France in 1919. It was observed during the early stages of the clinical trial that increased white blood cell count was present, however those patients who were able to survive more than a few days were transiently observed to have a severe decrease in white blood cell count. Due to the toxicity, mustard gas in its current form was not utilizable for clinical use. Despite this, potential clinical properties were indeed present within the agent (Faguet, 2008).

It would not be until World War II in which further research would be conducted on the properties of mustard gas. This time the research would be carried out by a research team lead by the pharmacologist Dr. Alfred Gilman and co-led by the pharmacologist and physician Dr. Louis Goodman in the United States of America by the Yale University funded by the US Office of Scientific Research and Development. During this research, animal experimentation was conducted on mainly mice which were at the time affected by lymphomas. After exposure to the agent, it was observed that there was an absolute decrease in the size and hardness of the lymphoma, indicating a decrease in the oncogenic properties of the tumour. These results prompted further research and clinical trials for humans were on the horizon. However, to conduct research and utilize the properties of sulphur mustard gas on humans, a less toxic form of the drug had to be synthesized. Dr. Alfred Gillman hypothesized that it was the electrophilicity of the agent which was responsible for the high toxicity of the agent due to its high chemical reactivity towards electron rich groups. Therefore, by reducing the electrophilicity of the agent, a less toxic form of the drug may be obtained. To synthesize a less electrophilic agent with similar properties, analogues of the

sulphur mustard gas were created, the most notable of which was the replacement of the sulphur functional group with nitrogen, thus leading to the creation of the so-called nitrogen mustards. These first agents created were the aliphatic group of agents and do not yet include our agent of subject, chlorambucil. (Fauget, 2008).

As Dr. Alfred Gillman had correctly hypothesized, the replacement of the sulphur functional group with nitrogen resulted in a less toxic form of the agent with a lower therapeutic index in humans. The team was then given the go ahead to begin conducting clinical trials on humans.

The first example of human experimentation utilizing the nitrogen mustards was conducted by the Yale research team in 1942 when a patient of 42 years old who was in seemingly critical condition due to the lymphoma present, was given 10 consecutive doses nitrogen mustard at 100 micrograms to 1 milligram per kilogram of body weight, intravenously. This is, on average, now two and a half times more than the regular dose given today. However, due to the highly experimental nature of the test, it was unknown how large of a dose should be administered to the patient. As expected from the research conducted on mice, the tumour masses had already began decreasing in mass and by the second day and by the end of the treatment trial the tumour masses had disappeared completely. A month later, unfortunately the patient had relapsed and was once again suffering from Hodgkin's lymphoma. Subsequent clinical trials performed on this patient were not as successful as the first trial; indicating a decrease in efficacy of the drugs in transient treatments. Despite the lacklustre results of the proceeding trials, this had not discouraged the scientist within the research group as they were convinced that their clinical trial was proof that cancer can be treated by pharmacotherapeutic methods and thus chemotherapy was created.

The dramatic but highly variable response of experimental tumours in mice to the treatment prompted the first test in humans later that year. The use of methyl-bis(b-chloroethyl) amine hydrochloride - the first approved alkylating agent and methyl-tris (b-chloroethyl) amine hydrochloride for Hodgkin's disease lymphosarcoma, leukaemia and other malignancies resulted in striking but temporary dissolution of tumour masses. Other agents included

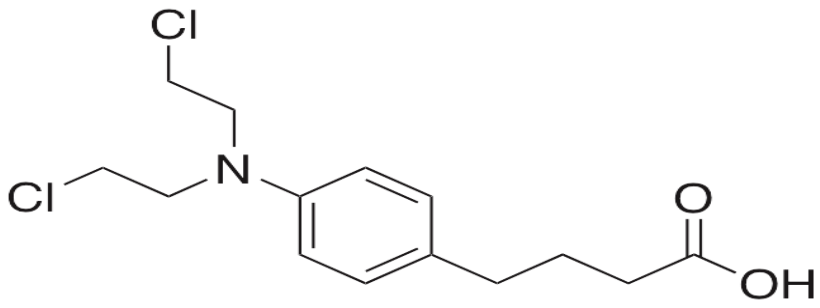
cyclophosphamide (the most widely used alkylating agent of modern times), uramustine or uracil acid, melphalan, ifosfamide, bendamustine and chlorambucil.

The success of the first human trial prompted further research and a further sixty-seven patients were provided for further clinical trials, in which the same results were observed and the correct dose 100 micrograms per kilogram of body weight was obtained. It must be noted that all these clinical trials were kept as a secret from the public and no information would be available on the subject until 1946.

The promising results of the research carried out by the Yale University research team prompted worldwide investigations into the properties of other alkylating agents and their effects on the oncogenic diseases. It would not be until the 1950's in which research began on the effects of aromatic mustards on oncogenic diseases and this is where our pharmacological agent, chlorambucil would come into the picture. (Curtis, 2005, Rhoades 1978).

Chlorambucil is a bilateral alkylating agent which was synthesized by Everett et al. (1953) and was approved for clinical use in 1957 in the United Stated of America (NCATS, no date). Chlorambucil is synthesized from 4-phenylbuanoic acid which undergoes nitration in the presence of concentrated nitric acid in acidic conditions to produce 4-4-nitrophenylbutanoic acid. 4-4-nitrophenylbutanoic acid then undergoes reduction in the presence of palladium or calcium carbonate to produce 4-4-aminophenylbutanoic acid. 4-4aminphenylbutanoic acid then reacts with 2 mol of ethylene oxide to get 4-4-bis-2-hydroxyethlyaminophenylbutanoic acid. 4-4-bis-2-hydroxyethylaminophenylbutanoic acid is then chlorinated in the presence of thionyl chloride to produce chlorambucil, the structural formula of which can be seen in **Figure 1** below.

Figure 1 – Structural Formula of Chlorambucil



Source: (2021). *https://drugs.ncats.io/substance/18D0SL7309*.

Chlorambucil is available under the brand name of Leukeran® and while it is not officially approved by the Food and Drug Administration for use in animals it has been prescribed in off label or extra label, usage in dogs cats and horses for treatment of immune mediated disorders and cancers. More specifically in dogs it is utilized to treat lymphomas, lymphoreticular neoplasms, chronic lymphocytic leukaemia macroglobulinemia and inflammatory bowel disease. It has also been found to be able to treat pemphigus complex when utilized in combination with prednisone. In cats chlorambucil is used to treat specifically chronic lymphocytic leukaemia, lymphocytic plasmocytic enteritis, pemphigus, inflammatory bowel disease and eosinophilic granuloma complex. In horses it has been mainly utilized for treatment of lymphomas. Chlorambucil is normally not utilized in ruminants. In humans chlorambucil has been mostly replaced by fludarabine for treatment especially that concerning younger patient. However, it is still widely utilized due to its well tolerability for the treatment of Hodgkin's disease, non-Hodgkin's lymphoma, chronic lymphocytic leukaemia, testicular, ovarian and breast cancer. As can be seen from the statements above, chlorambucil is used primarily to treat those cancers concerning the lymphatic system, blood and bone marrow, however the effects of chlorambucil on mammary gland cancer will be of primary concern during this thesis. (Dziuk, in Encyclopaedia of Toxicology (Second Edition), 2005).

2.2 Pharmacokinetics and Mechanism of Action

Chlorambucil is administered orally, normally using a 2 milli gram brown film coated tablet combined with inactive ingredients such as colloidal silicone oxide and microcrystalline cellulose. Moreover, oral oil suspensions are also available and are more often administered in cats than in dogs. The administration dose of chlorambucil must be determined from the patient's weight, body condition, general health condition and the type of cancer that is to be treated. Care must be taken to properly complete the treatment in order to prevent relapse. In general, the dose for dogs most commonly prescribed is 200 micrograms per kilogram body weight orally for 14 days; followed by 100 micrograms per kilogram of body weight. Tapering doses with prednisone may also be applied. It cats the dose most commonly prescribed is 100 to 200 micrograms per kilogram orally daily, the veterinarian will be responsible in this case in determining the duration of the treatment or alternatively 250 micrograms to 500 micrograms per kilogram of body weight every 48 to 72 hours has also been prescribed. Since the administration is done orally, it is normally done 1 hour before or 2 hours after eating. (DailyMed - LEUKERAN- chlorambucil tablet, film coated, 2021).

Chlorambucil is absorbed in the gastrointestinal tract and reaches peak plasma concentration levels within an hour and is transported by via the binding to the plasma protein albumin. Chlorambucil is a weak acid that is highly lipophilic and so may be taken up in the cells by passive diffusion. Due to its weak acidity, it may be ionized in acidic conditions and therefore its uptake is preferred in neutral intracellular spaces. It has been noted that the extracellular pH of tumour tissue is significantly lower than that of normal tissue and therefore this extracellular acidity enhances the uptake of chlorambucil; increasing the free acid amount. It is when chlorambucil reaches the liver and is converted into phenylacetic acid mustard that its main anti neoplastic affects will be expressed. Chlorambucil and its metabolites are transiently excreted via the urine (Humans, 2021).

As mentioned previously chlorambucil is an aromatic nitrogen mustard derivative and an alkylating cytotoxic agent, this means it is able to crosslink with DNA by forming covalent bonds with the nucleic acids and interfere with DNA replication preventing the production of antibodies as well as inducing cellular apoptosis by the accumulation of p53 and

subsequent activation of bcl-2 like protein or BAX protein which is the regulatory protein of apoptosis and is able to produce apoptosis by the piercing of the other membrane of the mitochondria (Humans, 2021).

Tests have been performed in order to observe the effects of chlorambucil on DNA. During these test hydrolysed DNA incubated with chlorambucil and DNA of cells in a free system also incubated with chlorambucil were analysed using high power liquid chromatography. Two peaks of radioactivity were observed during the experiments allowing for the observation of two or in some cases more adducts. For this reason, further experiments were conducted in which chlorambucil was incubated with DNA from cells that had been exposed to radiolabelling. From this experiment it was found that DNA which contained adenine gave rise to two peaks while those that had contained guanine only gave rise to a single peak of radioactivity and those which contained cytosine and hydrolysates prepared from labelled DNA and which had been incubated with chlorambucil alone did not indicate any adduct formation. From the experimentation performed conclusive evidence could be drawn to show that the major adducts induced by chlorambucil binding to DNA are guanyl adducts however a high quantity of adenylyl adducts may also be produced (Humans, 2021).

The common binding sites of chlorambucil once present inside the cell and exposed to the DNA include predominantly the N7 positioned guanine but may also bind to N3 positioned adenine, as well as thiol groups and other protein peptides. Several reasons for developing resistance have been observed in chlorambucil for example, the binding of chlorambucil to the thiol groups has been linked with the decreased efficacy of the drug as it may cause reaction of thiol groups with glutathione leading to the exportation of the conjugate by multi drug resistant proteins. The presence of an abundance of glutathione S-transferase will also lead to a much higher conjugation rate to glutathione and the transient release of them from the cell, resulting in higher drug resistance. Substantial metabolism of chlorambucil to phenylacetic acid mustard also have been shown to increase resistance to chlorambucil. (Trakoli, 2012) .

The binding of chlorambucil to DNA strands will prevent the separation of the DNA during mitosis thus interrupting this process from taking place (Roberts, 1975), an effect which is

of particular importance when preventing the spread of cancer cells. **Figure 2** below depicts information regarding the application of chlorambucil in different species for its most common diseases.

Figure 2 – Application of Chlorambucil in Different Species

Chlorambucil Dose	Other drugs	Species	Disease	Survival Time	Reference
200mg/kg		Dog	Naturally Occurring Tumours	153 days	Purdue University 2012
200mg/kg	Prednisolone 40mg/m2 q24h for 7 days then 20mg/m2 48hr	Dog	Chronic Lymphocytic Leukaemia	12 Months	BSAVA Small animal formulary 6th edition
200mg/kg	Prednisone 2.22mg/kg	Dog	Pemphigus Body		Animal Dermatology Clinic, Tustin, CA 92780, USA 2003
20mg/kg	Prednisolone 20mg/kg	Horse	B-Cell chronic Lymphocytic Leukaemia	6 Weeks	AVMA 2021
0.2-0.4 mg/kg		Humans	Hodgkin's Lymphoma	5 Years	Cancer.Org 2021
0.2mg/kg every 24 hours for 7 days then 0.1mg/kg	Prednisolone 40mg/m2 q24h for 7 days then 20mg/m2 48hr	Cats	Chronic Lymphocytic Leukaemia	14.4 Months	BSAVA Small animal formulary 6th edition
15-20mg/m2 q2wk	Prednisolone 40mg/m2 q24h for 7 days then 20mg/m2 48hr	Cats	Lymphoma	4-12 months	BSAVA Small animal formulary 6th edition
1-2mg/m2 p.o. q24h.		Cats	Immune mediated disease		BSAVA Small animal formulary 6th edition

2.3 Side Effects of Chlorambucil

Perhaps the most adverse and contraindicative side effect of chlorambucil is its carcinogenicity. To provide a proper understanding of the carcinogenic effects, several tests were performed on lab mice and rats. Both male and female rats were tested via intraperitoneal injection whereas only females were incubated with the drug via gavage. The tests carried out mainly by (Shimkin et al., 1966; Weisburger et al., 1975; IARC, 1981b) and (Berger et al., 1985; Cavaliere et al., 1990) provided evidence which showed that chlorambucil had and increased production of tumours in the lungs and haematopoietic system of rats and mice, whereas rats also had an increase in nervous system tumours. Increased incidences of tumours in the mammary glands and lymphomas in female mice and rats was also observed.

Equally as problematic to the increased tumour rate is the adverse effect of chlorambucil on bone marrow resulting in bone marrow suppression, therefore it is stated that the use of chlorambucil is contraindicated in patients with bone marrow depression or infection. Bone marrow suppression will lead to a vast amount of secondary side effects including anaemia, neutropenia, thrombocytopenia, leukopenia, and pancytopenia. Bone marrow suppression brought about by chlorambucil may be reduced and negated by proper withdrawal of the drug, however care must be taken in the correct time to withdraw treatment in order to complete the proper chemotherapy procedure while also minimizing the adverse side effects of the drug.

Other more common but less adverse side effects of chlorambucil are not unlike many other agents utilized for chemotherapy and include basic gastrointestinal disorders such as vomiting diarrhoea and nausea as well as a decrease in appetite, neurotoxic effects on the central nervous system including myoclonus, ataxia, twitching, flaccid paralysis, agitation, hallucination, and tonic-clonic seizures. (Cancer Research UK, 2019)

Dermatological side effects are also present as a result of chlorambucil treatment. Skin hypersensitivity reactions resulting in rashes which progress to erythema has been rarely reported but present. Urticaria and angioneurotic edema have been found to be produced

after subsequent treatment of chlorambucil. However, most commonly observed is alopecia and the delayed growth of cut fur. It is believed that this side effect is most prominent in the canine species of Poodles and Kerry Blues, both of which contain thick, rough curly coating.

While not normally present; incidences of hepatotoxicity, jaundice, pulmonary fibrosis, interstitial pneumonia, infertility in males and cystitis have been reported in canine species. No reports could be found on these particular side effects in feline species with the exception of hepatotoxicity (Ware, 2017).

2.4 Chlorambucil in Experimentation

The objective of this portion of the thesis is analyze and review several studies carried out utilizing chlorambucil with regards to mammary gland and its potential carcinogenicity in order to draw conclusions for its utilization in veterinary medicine.

2.4.1 Chlorambucil Carcinogenesis in BALB/c Mice (Oral Administration):

For our first review we will be looking at an experimentation carried out by the Institute of Pathological Anatomy and Histology, Division of Cancer Research and Department of Hygiene of Perugia University (Cavaliere, Pietropaoli, Alberti and Vitali, 1990).

During the experimentation, BALB/c lab mice of both sexes were utilized in order to measure the level of carcinogenicity present within chlorambucil. During the experimentation, lab mice which had been bred at the Division of Cancer Research Institute of Pathological Anatomy at Perugia University in Italy, were used and divided into two groups. Group 1 contained 53 males and 54 females and received a dose of chlorambucil in aqueous suspension perorally via gavage at a dose of 1mg/kg bodyweight. It was administered at a rate of five times per week for twelve weeks. Group 2 was the control group and contained 50 males and 50 female mice which had only received sterile saline solution orally during the same administration period. Both groups were housed in metal cages and were kept under identical environmental conditions and fed the same diet. All

animals where inspected daily and those found to be in morbidum were autopsied and examined immediately.

It had been observed that the survival rate in both sexes during the experimentation had been reduced by a P value of less than 0.001. During the experimentation a significant increase in the chance of obtaining lymphoreticular tumours were present at a P-value of less than 0.01 in males and less than 0.001 in females. There was also an increase in the chance of obtaining lung tumours at a P-value of less than 0.001 in both males and females.

With regards to mammary gland cancer, in the female lab mice during the experimentation an increased incidence of mammary carcinomas was observed at a P-value of less than 0.05. A conclusion for the carcinogenic and mutagenic effects of chlorambucil on lab mice could be drawn from these results indicating increased incidences of certain types of cancer with sex related predisposition for certain types and the potential for these detrimental effects to be present within other species. A total of five mammary gland tumours were observed during the experimentation, three adenocarcinomas type B and one adenoacanthoma were found in treated female mice in group 1 whereas only one adenocarcinoma type B was observed in the control group which was found in a 153-week-old female.

2.4.2 Prospective Trial of Metronomic Chlorambucil Chemotherapy in Dogs with Naturally Occurring Cancer:

The following experiment was carried out by the Department of Veterinary Clinical Sciences, Purdue University School of Veterinary Medicine, West Lafayette, IN, USA (Leach et al, 2011).

Metronomic chemotherapy is a form of therapy which utilises low doses of the chemotherapeutic agent applied normally over a long period of time without allowing for extended periods of rest. The primary mechanism of action for metronomic chemotherapy is believed to work by antiangiogenic effect which is achieved either via the direct inhibition or killing of endothelial cells in tumour vasculature, stimulation of the immune system, killing of bone marrow derived endothelial progenitor cells or directly inhibiting tumour

cells via drug driven effects and inhibiting a specific target when targeting drugs were used in addition to metronomic therapy (Mross and Steinbild, 2012). The use of metronomic therapy in veterinary medicine is based upon the antiangiogenic effects and mainly includes COX-antagonists. Cyclophosphamide is the most commonly utilised agent for low dose metronomic chemotherapy (European Journal of Cancer, 2013) and is normally used in conjunction with other agents. Its use in several studies conducted on a number of different species with varying grades of cancer showed that it is clinically beneficial in treating a wide range of cancer. (Lien et al, 2013). Unfortunately, several side effects are associated with the use of cyclophosphamide, most notably urinary cystitis which can be present in a severe haemorrhagic form (Arch int-med vol 136, May 1976). Therefore, the application of other drugs such as chlorambucil for metronomic therapy which are present with lesser amounts and severity of side effects are of particular interest for this form of chemotherapy. While chlorambucil may not be as effective as cyclophosphamide for metronomic therapy, since chlorambucil does not present the same nephrotoxic or gastrointestinal side effects as cyclophosphamide, its use in patients presenting symptoms of these diseases may be permitted where cyclophosphamide would only exacerbate these problems.

The main goal of the experiment was to observe the toxicosis and anti-tumour activity of chlorambucil within dogs particularly from the metronomic aspect of treatment. This form of treatment is of particular interest during the investigation of chlorambucil and other chemotherapeutic agents as it has shown to work through two mechanisms of action in which it may modulate the immune response by depleting immunosuppressive regulatory T-lymphocytes and also prevent angiogenesis. Metronomic treatment has been shown (primarily in human experimentation) to extend the time of disease progression of some cancer types while also reducing the risk of causing treatment induced adverse effects.

Before proper experimentation began a pilot study had been conducted which contained six dogs with histologically diagnosed cancer. Initially an oral dosage of 2mg m-2 had been administered daily to the dogs and the affects were observed. These results were encouraging, showing delays in disease progression. Once cancer had begun to progress again the dosage was increased to 4mg m-2 daily, and due to their transiently being no

observation in the increase of drug toxicity, the dosage of 4mg m- was determined to be the satisfactory dose to continue on with proper experimentation.

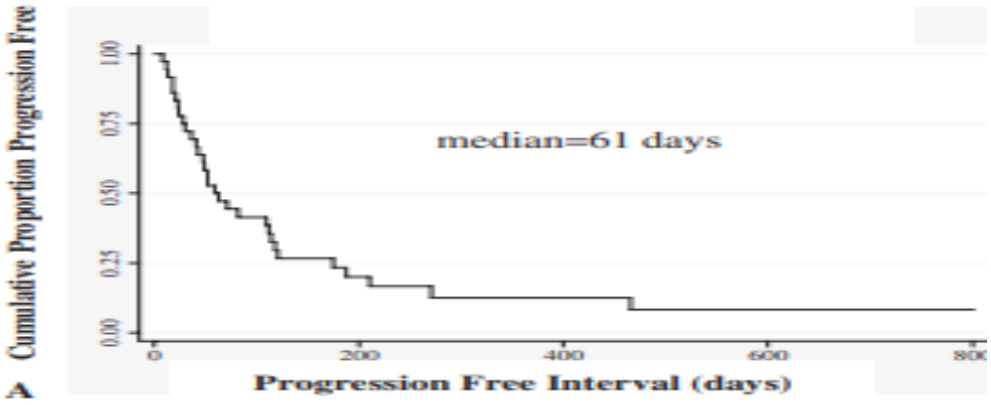
36 dogs had been selected for the clinical trial and eligibility criteria included being histologically diagnosed with measurable cancer which had failure of any prior therapy and an expected survival time of less than six weeks. Due to the belief that metronomic therapy has an effect against a wide variety of cancers the trial was open to dogs containing any type of cancer. Chronic blood count, biochemical profile tumour measurements and tumour staging was performed prior to the clinical trial. The physical examination and chronic blood counts were repeated at two and four weeks then at four to six-week intervals. Tumour measurements and imaging were performed at four weeks and then at four to six-week intervals. Tumour response was assessed using the response evaluation criteria in solid tumours and using volume measurements.

Chlorambucil was administered orally at 4mg m-2 once daily, for dogs weighing more than 8 kilograms. The dose was rounded to the nearest 2 milligrams in order to match dosing with commercially available 2mg tablets. For dogs weighing 8 kilograms or less the chlorambucil was compounded at 4mg m-2 to obtain precise dosing.

The response to the therapy depending on the tumour type is summarized by the **Figure 3** (reproduced from the study) as follows:

Figure 3 – Experiment Data Summary

Tumour type	Number enrolled	CR	PR	SD	PD	PFI (weeks)	Survival (weeks)
Haemangiosarcoma	5	0	0	3	2	3, 6, 16, 17, 27	3, 7, 20, 35, 37
Fibrosarcoma	4	0	0	1	3	1, 3, 3, 16	1, 21, 26, 29
Mast cell tumour	4	1	0	2	1	3, 9, 23, 67	6, 9, 23, 98
Soft tissue sarcoma	3	1	0	1	1	3, 17, 35	3, 30, 35
Anal sac adenocarcinoma	3	0	0	2	1	2, 10, 30	2, 10, 63
Osteosarcoma	2	0	0	2	0	8, 16	10, 22
Histiocytic sarcoma	2	0	1	0	1	2, 39	2, 40
Haemangioma	2	0	0	1	1	7, 10	10, 69
Myxosarcoma	1	0	0	1	0	7	9
Leiomyosarcoma	1	0	0	0	1	4	4
Squamous cell carcinoma	1	0	0	0	1	5	56
Hepatocellular carcinoma	1	0	0	0	1	6	9
Thyroid carcinoma	1	1	0	0	0	114	114
Oral carcinoma	1	0	0	0	1	4	4
Pulmonary carcinoma	1	0	0	1	0	25	53
Rectal carcinoma	1	0	0	1	0	12	12
Malignant melanoma	1	0	0	0	1	7	66
Chemodectoma	1	0	0	1	0	75	75
Nephroblastoma	1	0	0	1	0	7	45



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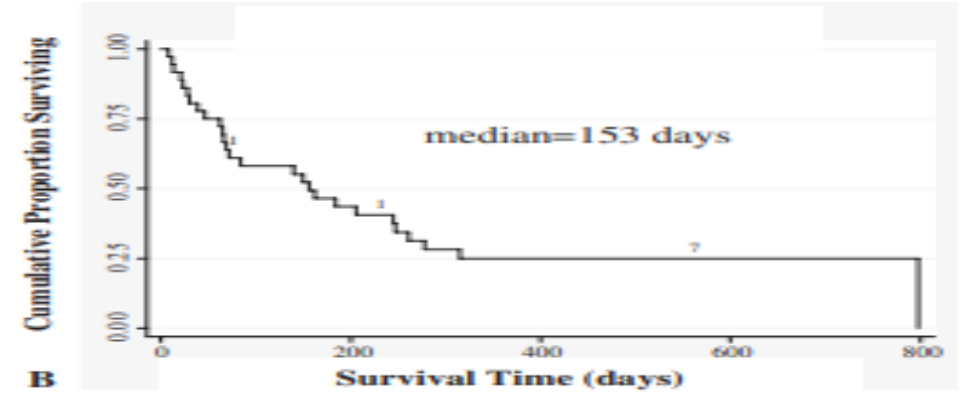

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Source: (Leach et al, 2011)

The clinical trial was well tolerated from a toxicology point of view as toxicoses was uncommon and only limited to first and second grade gastrointestinal toxicity in four dogs, while no grade three or four toxicosis was noted.

The clinical trial yielded positive results as the median of the progression free interval was 61 days and that of the survival time was 153 days successfully extending the time taken for disease progression to occur. A complete remission was achieved in 3 dogs and lasted over 35 weeks, those dogs contained a mast cell tumour, soft tissue sarcoma and thyroid carcinoma. Partial remission was observed in a single dog with histiocytic sarcoma which had a duration of 39 weeks. Finally, stable disease was noted in 17 dogs with varied different types of cancers.

The experiment gives knowledgeable insight into how metronomic treatment may also be applied during mammary gland cancer to obtain higher success rates of treatment or even just to prolong the life of the animal being treated while greatly reducing the risks of toxicity caused by the veterinarian.

2.4.3 Chlorambucil and prednisolone chemotherapy for dogs with inoperable mast cell tumours: 21 cases:

The following experiment was carried out by The Queen's Veterinary School Hospital, Department of Veterinary Medicine, University of Cambridge (Taylor, Gear, Hoather and Dobson, 2009).

In the following experiment canine patients were selected which contained mast cell tumours that were unable to be treated by radiotherapy or surgery. The experiment contained a total of 21 dogs which were selected from the Queen’s Veterinary School Hospital in the

University of Cambridge. Out of the 21 patients, 6 contained high grade tumours while 13 had intermediate grade tumours and the remaining 2 patients were only diagnosed by cytology (non-gradable tumour). The experiment contained 12 males and 9 females, 7 of which had been neutered from each group. The breeds included in the experiment were 9 Labrador Retrievers, 2 cross-bred dogs as well as 2 Stafford Shire Terriers, and 1 Boxer, Dachshund, Shar Pei, West Highland Terrier, English Springer Spaniel, Greyhound and Jack Russel respectively. The patients ages ranged from 4 to 13 years, with the mean age being eight years. The weights of the patients ranged from eight kilograms to thirty-eight kilograms and eight hundred grams with the mean being twenty-five and a half kilograms.

During the experiment, the patients were set to a chemotherapy protocol which included a dosage of 5mg/m2 of chlorambucil administered orally every other day; as well as starting dose of prednisolone of 40mg/m2 orally once daily for fourteen days, which was subsequently reduced to a dosage of 20mg/m2 daily every other day. During the experimentation if complete remission of the patient had occurred then the treatment was discontinued after six months. However, in patients not showing complete remission the treatment was applied continuously. The patients were assessed after the first two weeks of experimentation followed by scheduled monthly checkups. If the patient's condition became more pathological, then more frequent evaluation was performed.

The interpretation of the results was evaluated by the measurement of the tumours sizes as well as radiological, cytological and ultrasonographical examination if the oncologist deemed fit. A hematology profile of the patients was also taken into account every four weeks in order to observe the presence of myelosuppression. This together with any other noticeable defects were graded accordingly. A timeline of the progressive effects of the treatment was conceptualized by the utilization of a survival time interval, the time present from the start of the experiment till the death of the patient, and a progression free interval, the time present from the start of the experiment till the progression of the tumours such as an increase in size.

The results of the experiment do not seem to have been affected by previously mentioned factors such as the age or weight of the animal as well as the fertility status of the dog as

well as interestingly the stage of the tumour, recurrence as well as presence of metastasis. The location of the tumours was also taken into account when determining the results and seems to point at it being a factor when determining survival rate as those with tumours on the head had the longest survival time interval of an average of five hundred and fifty three days, compared to those with tumours present on the inguinal canal which had a much poorer survival time interval of only an average of sixty one days. However, due to the lack of proper sampling size no significant statistical conclusion may be drawn from these results but it may point to important information and deciding factors when considering the survival time and therefore the effectiveness of patients undergoing this specific chemotherapy. These results indicate whether or not the treatment method is worth performing in case of mast cell tumours present within the mammary glands. However, further experimentation with specific importance given to the location of the tumour is required.

The results observed are as follows; fourteen percent of the patients were noted to have a complete remission of their mast cell tumours while twenty nine percent of the patients had a partial remission of their mast cell tumours resulting in an overall response rate of thirty eight percent. The median progression free interval for those animals which showed a response to the treatment was five hundred and fifty-three days; with the progression free interval of the patients including those which did not show a response to treatment being one hundred and sixteen days. The median survival time interval of the patients in the experiment was one hundred and forty-three days. By the end of the study five dogs were still alive, fifteen were euthanized due to the progression of their disease, two others were euthanized due to other reasons, and one was stated to be lost due to “follow up”. None of the patients exhibited any hematological toxicities.

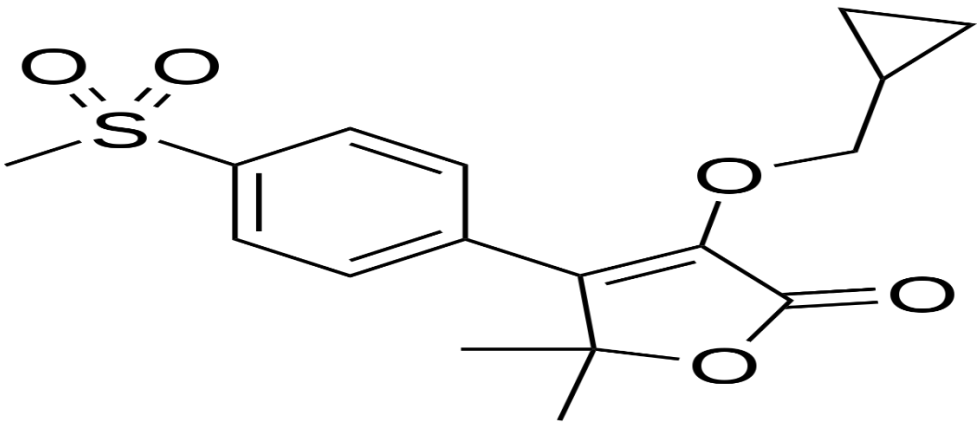
3.0 Firocoxib

3.1 History and Introduction

Firocoxib is a drug which is currently available for veterinary use under the brand names Previcox and Equioxx in dogs and horses respectively (Gollanker, 2018). No studies have been carried out for its current application in humans while only a few tests have been done for its use in other animals such as cats. Therefore, currently there is no full FDA approval for firocoxib in other species.

Firocoxib contains the chemical formula of $C_{17}H_{20}O_5S$ and has a molar mass of 336.40 $g \cdot mol^{-1}$. Firocoxib belongs to the pharmacological group of non-steroidal anti-inflammatories or NSAIDS for short and belongs to the COX-2 inhibitor class of this group. Firocoxib contains two polymorphic forms, known as an A form and a B form. However, during manufacturing only the B form is produced. It is used to treat inflammation and pain present in osteoarthritis and may also be used as a secondary choice to treat pain and inflammation present after surgery or trauma. (Hanson et al, 2008) While instances of the use of firocoxib to treat cancers such as transitional cell carcinomas in dogs have been reported, the use of firocoxib for these types of treatment has not been fully studied or approved, so much like the previously discussed chlorambucil, the use of firocoxib for treating cancer must be stated as an extra label or off label use of the drug. It is available in a tablet and oral paste form as well as a compound liquid form.

Figure 4 – Structural Formula of Firocoxib



Source: wikipedia.org/wiki/Firocoxib

Comparatively to a lot of other pharmacological agents the history of firocoxib is relatively young. Our first encounter with properly defined NSAIDs begins in 1897 with the invention of Aspirin by Felix Hoffman of the Bayer company, however the technology to understand the mechanism of effect was not available at the time. It was not until 1971 when Sir John Vane discovered that Aspirin worked by inhibiting the production of the pro-inflammatory agents, prostaglandins (El-Bogdadi, 2021). This discovery allowed for the production of further NSAID agents such as ibuprofen. In 1976 the first cyclooxygenase enzyme or COX-1 was purified and the mechanism by which the production of inflammatory mediators was produced was being more clearly understood Cyclooxygenase enzymes are responsible for catalysing the production of the previously mentioned prostaglandins as well as thromboxane and levuloglandins (Botting, 2010).

Since the discovery and isolation of the COX-1 enzyme as being the target enzymes when trying to prevent inflammation, numerous NSAIDs have been manufactured since the seventies with more specific and targeted effects. However, these agents acting as COX-1 inhibitors while having less severe side effects than steroidal anti-inflammatories were still not without their own problems. COX-1 enzymes are responsible for production of prostaglandins and thromboxane in multiple types of cells but mainly in gastro-intestinal tract cells and in platelets. This unfortunately results in side-effects commonly effecting these areas such as stomach ulceration and bleedings and aplastic anaemias. Therefore, the search and need for less harmful anti-inflammatory drugs was still procurable. (Marnett and Kalgutkar, 1999).

While suspicions and evidence to the existence of a COX-2 enzyme was present for some time, it would not be until 1991 where the existence of the COX-2 enzyme was finally able to be fully confirmed by a Dr. Dan Simmons of Brigham Young University when he was able to clone the enzyme. COX-2 enzymes are responsible for the production of prostaglandins in the inflammatory cells themselves as well as the central nervous system, therefore there would be no loss of gastrointestinal cell structure as well as ulcer and bleeding formation that is present within the COX-1 enzyme inhibitors. Since the discovery of COX-2 enzymes the shift in manufacturing of NSAIDs which targeted the COX-2 enzyme instead of the COX-1 enzyme was prompted. (Marnett and Kalgutkar, 1999).

Firocoxib was first manufactured by the Merial company which has now been merged and is a part of the Boehringer Ingelheim Vetmedica GmbH company. Firocoxib was a drug first approved in the United States market in 2005, whereas use in other continents began as early as September of 2004. It was marketed under the brand name Previcox and was sold mainly as an oral paste targeted towards horses but was also approved for use in dogs. In 2011 and injectable IV form of the drug was also approved (Davis, 2018). In 2016 a rebranding of the drug took place. The oral paste form of the drug which was to be mainly utilised in horses was rebranded to Equioxx while the tablet form of the drug which was mainly used in canines kept the original brand name of Previcox. (Nesson, 2017).

3.2 Pharmacokinetics and Mechanism of Action

As previously mentioned, Firocoxib is an NSAID belonging to the COX-2 inhibitor class, therefore anti-inflammatory as well as analgesic effects are produced by the inhibition of the production of prostaglandins from arachidonic acid into prostaglandin H (Chan et al., 1999; Garavito & Mulichak, 2003). In vitro assay tests have been performed to show that Firocoxib is more COX-1 sparing when compared to traditional NSAIDS and the ratio of COX-1 : COX-2 is greater than what is present in other drugs thus classifying firocoxib as a selective COX-2 inhibitor. The inhibition of COX-2 is of particular interest when looking at treatment for cancer and more specifically malignant mammary neoplasia within canines as this type of cancer (along with others) results in an over expression of COX-2 which leads to severe inflammation and a resultant poor prognosis of the animal. Therefore, inhibition of COX enzymes during cancer treatment can result in a prolongation of the patients suspected life span. (Papich, 2016).

The recommended doses for firocoxib when given orally is 5mg/kg every 24 hours in dogs (normally given in tablet form), 0.1 mg/kg every 24 hours in horses (normally given as a paste) and while not yet approved for cats a dose between 0.75-3 mg/kg every 24 hours is estimated to be used.(Kvaternick et al, 2008).

The absorption of firocoxib is rapid with it being detectable within the plasma as early as fifteen minutes and peak plasma concentration being reached as early as one hour to as late as ninety minutes in dogs and up to four hours in horses. Firocoxib remains within the plasma for twenty-four hours which allows for daily dosing to be applied. The bioavailability has been demonstrated to be 36.9% in dogs, with a proportional relationship between administration dose and plasma concentration. Tests done on dogs receiving firocoxib for seven days showed that a steady state in concentration was achieved within three days. It was also stated that feeding was unlikely to affect the bioavailability of firocoxib. (Talcott and Gwaltney-Brant, 2013).

Within the same test the distribution of firocoxib within canines was measured by the utilisation of radiolabelling technology. Within six hours following oral administration of firocoxib the highest concentration of residues of the drug were found within components of the digestive tract, that being the stomach, small and large intestines and within the bile. Residues found within the remaining organs and fluids were unremarkable. It took up to three days for the mean concentration of the radioactive compounds within the organs and fluids to return to normal. The calculated half-life for firocoxib is seven hours and forty minutes in dogs, eleven hours in foals, thirty to forty hours in adult horses and between nine and twelve hours in cats. It must be stated when discussing the distribution of firocoxib that it has a very high affinity for being bound to plasma proteins with an estimated range for 96-98% and therefore interactions with other substances which contain a similar affinity for plasma protein binding such as anticoagulants or even other NSAIDs will occur and therefore must be taken into consideration when utilising the administration of firocoxib alongside these other agents. (Papich, 2016).

The metabolism of firocoxib while having similarities also contains differences, primarily concerning the method of excretion, between dogs and horses. In dogs, firocoxib undergoes metabolism primarily in the liver via the processes of dealkylation and glucuronidation. In vivo test performed via oral administration of firocoxib showed that elimination of firocoxib and its related metabolites are quite rapid with a mean half-life of 7.59 hours and effectively complete elimination 3 days after stopping administration. The primary route for excretion

within dogs appears to be through the bile duct within the gastrointestinal tract, with the final route of elimination being via the kidneys. (European Medicines Agency, 2010).

Within horses, firocoxib is also absorbed rather rapidly via oral route. Within four hours the peak plasma concentration of f 0.075 µg/ml is achieved. The mean bioavailability of firocoxib following oral administration is 79%, much higher than the 39% seen in dogs. Tests utilising radioactive firocoxib which was administered daily for seven days within 11 horses showed the elimination half-life of a single dose to be 29.6 (7.5) hours. The tests also showed that the vast majority of the radioactivity was excreted within three days after the final dose was administered. In all, a total of 83.5% of the radioactive dose had been excreted, 68% of which had been excreted via the kidneys in the urine, and an estimated 15% had been excreted via the faeces. This method of excretion is in contrast with that found in dogs, as the primary method of excretion in horses appears to be via to urine as opposed to being through the bile. The metabolic pathways for firocoxib in horses are also dealkylation and glucuronidation. Within the kidney firocoxib metabolised to descyclopropylmethylfirocoxib, its dealkylated parent, and its glucuronide conjugates. It must be noted that the descyclopropylmethylfirocoxib metabolite was a major component found within the urine excrete. However, the amounts present within the faeces was unremarkable. The glucuronide conjugates and the dealkylated parents were notable components within both the faeces and the urine. (Kvaternick et al, 2007).

3.3 Toxicity, Side Effects and Precautions for Firocoxib

Toxicity studies for testing the single dose toxicity, repeated dose toxicity, embryotoxicity, and mutagenicity have been carried out; primarily on rats, mice, and rabbits. Few studies have been carried out to measure the actual toxicity present with dogs and horses.

Single dose toxicity tests performed showed that the acute toxicity potential for firocoxib is relatively mild as the acute LD50 for orally applied firocoxib was more than 2g/kg bodyweight in mice and rabbit and the acute LD50 of dermally applied firocoxib was also 2g/kg within rabbits. (European Medicines Agency, 2010).

When testing repeated dose toxicity, two tests were performed in rats and GLP or good laboratory practice tests were performed on dogs and in horses. In the first test performed on rats a daily dose of 0, 50, 150 or 500 mg/kg/bw/day of firocoxib was administered for two weeks, orally via a gavage. The first study found that the Albumin to Globulin ratio was significantly decreased at a dose of 500mg/kg bodyweight and further pathological investigations within this dose range showed significance in the decrease of the cardiac weight and showed an increase in the frequency of renal tubular cell vacuolation, hepatocyte vacuolation and follicular cell hypertrophy present within the thyroid gland. These changes were significantly reduced or not present at the administered doses of 150mg/kg/bw/day or lower. (European Medicines Agency, 2010).

The second study employed groups of rats consisting of twenty rats per group and lower dose rate of 0, 3, 30, and 60mg/kg body weight. However, the application of the drug was done over a much longer course of time - three months as opposed to two weeks. In doses of 30mg/kg body weight a significant increase in salivation in both sexes of rats was noted. Within female rats during the testing period, there was a significant and transient increase in the amount of food consumption and therefore weight gain at all dose levels. It was also noted that female rats receiving a dose of 60mg/kg body weight exhibited a significant reduction in overall body weight during the allotted four-week recovery period. No gross pathological changes were evident during necropsy study and while changes in biochemistry and haematology were present, the changes were often within control ranges and no clear dose relationship could be established. Histopathological studies revealed a significant increase in kidney weight at all doses treated and an increase in liver weight and hypertrophy of the thyroid follicular cells at the dose range of 30mg/kg body weight. Most of these changes were reversible after the four-week recovery period except for the liver weight present in high dose female recipients. These changes were suggested to show an adaptive change to treatment with utilisation of a microsomal enzyme inducer. (European Medicines Agency, 2010)

The repeated dose toxicity tests performed on dogs consisted of GLP studies. Doses ranging from 5mg/kg bodyweight to 15mg/kg bodyweight or higher were utilised during the test. The target organs for the test were the gastrointestinal tract, the liver, and the brain. The test

concluded that doses of 5mg/kg body weight was generally well tolerated and doses of 15mg/kg body weight or higher were associated with adverse side effects. It was also noted that enhanced toxicity was shown by firocoxib when administered to dogs less than 3 months of age. Pathological changes to the gastrointestinal mucosa were the most notable changes present within the study. Other changes included mild glossopharyngeal lesions, brain vacuolisation and changes to lipid metabolism. (European Medicines Agency, 2010)

The GLP studies performed on horses consisted of groups containing 6 horses per group and dose levels of 0.1 0.3 and 0.5mg/kg body weight per day for thirty days. Many changes occurred within the test subjects during the experimentation including oral lesions, reduction in post treatment heart rate. Also, many changes in biochemistry parameters such as decreased bilirubin and increased urea, chloride, and an increased incidence of nematode related lesions were noted. However, despite the changes listed; the overall end result of the experiment proved to be rather unfruitful as none of the previously mentioned changes could be related to a dose response relationship and therefore shown to be of dubious biological significance. The most notable change occurred at a dose of 0.5mg/kg body weight which resulted in the reduction of numerous red blood cell parameters. (European Medicines Agency, 2010)

Reproductive and teratogenicity tests were performed in both rats and rabbits; however, they were only performed for one generation therefore the results of the experimentation are still inconclusive. The tests showed that the toxic effects of firocoxib were more prevalent in rabbits than in rats. The studies also showed that firocoxib was both embryotoxic and fetotoxic in both rats and rabbits and was able to induce a variety of abnormalities and malformations in the visceral and skeletal components. Such changes included early lactation and feed reduction, reduction in gestation length and weight, a reduction in successful inseminations, a reduction in pup weight, litter size and survival rate, malformation of the pup's tales, an imperforated anus and heart vessel malformation recorded in one foetus which contained no aortic arch, no subclavian vessels, and no innominate vessels. Therefore, while the study is not entirely conclusive there is enough evidence present to suggest that the use of firocoxib in pregnant and lactating animals is contraindicated and therefore should not be used. (European Medicines Agency, 2010)

Mutagenicity studies for firocoxib were also performed however proved to be of no significant value as all the studies yielded negative results both in the presence and absence of metabolic activation. Currently there have not been any carcinogenicity studies carried out on firocoxib; however, there appears to be no structural alterations directly linked to this specific family of compounds. (European Medicines Agency, 2010)

Since firocoxib belongs to the COX-2 type inhibitors of the NSAIDs, a lot of the classical side effects that are normally associated with NSAIDs are either absent or reduced, mainly the gastrointestinal associated side effects, however this does not mean that gastrointestinal side effects are not present. Overall, the general consensus does seem to be that COX-2 inhibitors are generally safer for use than classical NSAIDs (Marnett and Kalgutkar, 1999).

The main side effects that are present in both forms of firocoxib (i.e. the tablet form and the paste form) include; vomiting, possible changes in bowel movements which result in diarrhoea which may contain black tarry or even bloody stools, jaundice, abnormal changes in behaviour mainly increased or decreased activity levels, incoordination, seizures and even aggression, changes in drinking habits such as the amount consumed and the frequency at which it is consumed which is most likely a secondary effect of the diarrhoea, changes in the frequency amount, colour and smell of the urine are also present, as well as changes present within the skin including redness, scabs and pruritis and finally it may also result in a sudden unexpected weight loss. (Drug FAQ's: PREVICOX, 2021).

Other side effects are prevalent firocoxib when the drug is used in the paste form, therefore Equioxx, as opposed to the tablet form. These side effects involve the oral mucosa and the surrounding tissues and include soreness and even ulceration present on the tongue and inside of the mouth with sores, scabs, and a general irritation of the facial skin; mainly around the mouth. These side effects are normally mild and do not usually require any further treatment in order to heal (European Medicines Agency, 2017).

Prior to receiving the medication in it highly recommended that measurements of the patients' complete blood count (CBC), kidney function, urinalysis, liver enzymes and electrolytes as well as a general physical examination are performed. It is also suggested that to repeat these tests as much as possible during the duration of the treatment. (Gollanker, 2018).

Firocoxib treatment should not be applied in animals with known hypersensitivity towards other NSAIDs, in breeding pregnant or lactating animals, in animals with stomach or gut disorders, bleeding disorders or in animals with reduced liver, heart or kidney function. Firocoxib should also not be used in dehydrated animals. It is also highly recommended to not use firocoxib in animals less than seven months of age and at a body weight of 5.750kg. (Gollanker, 2018).

Firocoxib also undergoes numerous drug interactions and therefore caution is required when administering this drug with other drugs. It should not be administered with other NSAIDs, corticosteroids, ACE inhibitor, digoxin, furosemide, fluconazole, nephrotoxic drugs, and drugs with high protein binding efficacy such as phenytoin.

3.4 Firocoxib in Practice

Within the process of cancer itself, COX-2 expression is seen in multiple types of cancer cells which play a role in proliferation, angiogenesis, metastasis, and inflammation of the cancer cells, essentially having a so-called cancer "stem cell like" activity. COX-2 is released into the tumour microenvironment via cancer associated fibroblasts and macrophage type 2 cells. COX-2 may enable cancer cell resistance to chemotherapeutic drugs via a COX-2 mediated hypoxia within the tumour microenvironment together with positive interactions between anti apoptotic mediators and the YAP1 protein coding gene (Hashemi Goradel et al., 2018). With regards to mammary gland cancer specifically, approximately 60% of canine mammary gland tumours result in an over-expression of the COX-2 enzyme with the majority of these tumours being classified as malignant therefore one can conclude that the higher the expression of COX-2 enzyme due to cancer, the poorer the prognosis of the patient (Queiroga, Alves, Pires and Lopes, 2007).

For the reasons mentioned above, the therapeutic use of COX-2 inhibitors, and in this case Firocoxib, has become of importance when mitigating the damage caused inflammation and metastasis of the cancer cells while also sensitising those some cancer cells to other forms of chemo or radio therapy.

3.4.1 Evaluation of COX-2 Expression in Canine Mammary Tumours and its Relation to NSAID Therapy

The first experiment we will be looking at was conducted at the University of Veterinary Medicine in Budapest by the Department of Internal Medicine in collaboration with the Veterinary Oncology and Haematology Centre (Sood, R., Vajdovich. P, 2013, *Evaluation of COX-2 expression in Canine Mammary Tumours and its Relation to NSAID Therapy,* University of Veterinary Medicine Budapest).

The goal of the experimentation was to conduct a study to evaluate the expression of COX-2 in canine mammary tumours and to provide therapeutic treatment after surgery utilising NSAIDs.

During the experimentation a total of 42 dogs were utilised, 2 of which were male. The age range of the dogs used for the experiment was as low as 4 years to as high as 15 years. During pre-examination it was determined that 28 of the 42 subjects contained a primary tumour and 15 had a secondary or relapsing tumour and one subject contained both. Once routine blood work, chest x-rays and ultrasonography had been performed, the tumours were excised during surgery and samples were made for histopathological examination.

NSAID therapy was provided post-surgery of which included piroxicam at a rate of 0.3mg/kg body weight orally which was given to 23 dogs, meloxicam given at a dose of 0.2 mg.kg body weight initially then followed by a 0.1mg/kg body weight dose twice daily for a day and then a further 0.1mg/kg body weight dose consecutively which was given to 18 dogs, and finally firocoxib which was given to 12 dogs and administered at a rate of

0.5mg/kg body weight daily. All the NSAIDs were provided for a four-month period and the results then evaluated.

It must be noted that during the experiment a further 13 dogs had received other forms of chemotherapy including doxorubicin, cyclophosphamide, and carboplatin, while four other dogs received a combination protocol of different drugs.

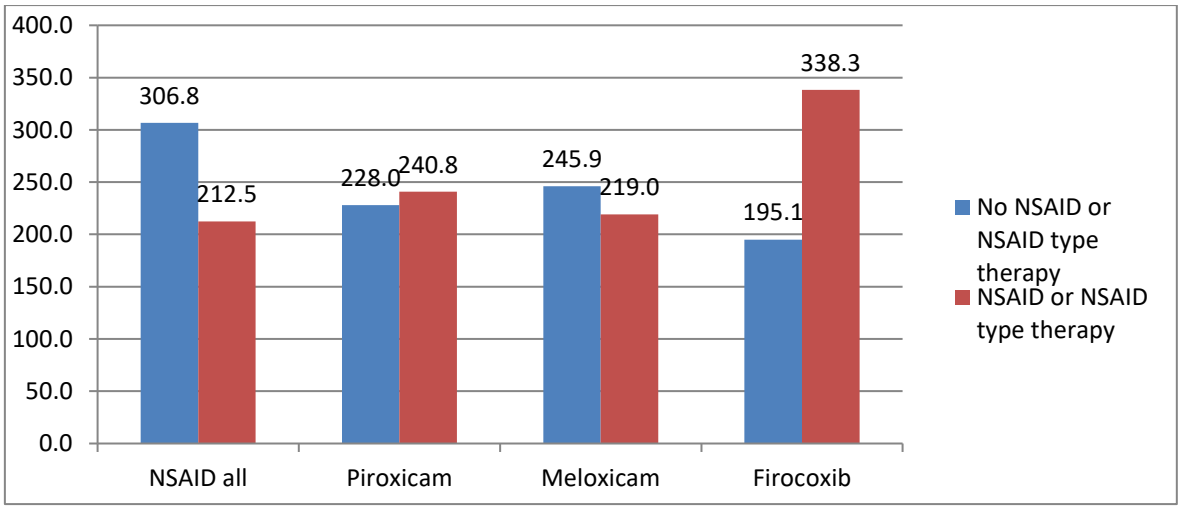
The samples collected were sent to the laboratory for proper evaluation. Queiroga et al was the method used to determine the COX-2 expression by immunohistochemistry; however, minor modifications were made. The scoring was done in a blinded and independent manner by two different investigators to provide a more distinguished array of results. A total of 1728 tissue array cores were used, stained via the labelled Streptavidin-Biotin method (LSAB). A scoring system of 0-3 was used for determining the range of cytoplasmic staining present within the tumour cells with 0 being the lowest and 3 being the highest. Over 90% of the staining's done had a strong intensity or a score of 3.

After the histopathological examinations were done, the results had revealed that from the samples provided, 13 of the tumours were benign while 29 of the tumours were malignant. Many different tumour types were revealed during the examination; the most notable of which was the simplex carcinomas - 8 of which were of the solidum type, another 8 which were of the tubulopapillare type and one which was of the anaplastic type. A further 8 tumours were of the carcinoma complex type. Present also was one carcinosarcoma, one fibrosarcoma, two chondrosarcoma, twelve lesions which were determined to either be adenomas, hamartoma or hyperplasia and one steatitis. Of the tumours determined from the samples the majority, a 48.27%, were of grade 1 malignancy, 24.13% were of grade 2 malignancy and 27.58% were of grade 3 malignancy.

The study concluded that the expression of the COX-2 enzyme was more pronounced within sarcoma and carcinoma subtypes of tumours when compared to benign forms. Those animals which expressed a COX-2 enzyme percentage of higher than 50% had a notably decreased in survival time compared to those with a COX-2 enzyme expression percentage of less than

50%, an average of 339.85 days in those with less than 50% compared to 88.556 days to those with more than 50%. The subsequent NSAID therapy was unsuccessful in prolonging the survival time of the patients, however, those patients which expressed a COX-2 percentage of higher than 50% did have a significantly higher survival time when treated with firocoxib (an R-value of 0,2 was determined) than those who received other forms of NSAIDs therapy (an R-value of 0 for piroxicam and -0.05 for meloxicam) or no NSAID therapy at all (R-value of -0.1 - -0.15) as shown in **Figure 5** below:

Figure 5 – Survival times (days) with different NSAID therapy



Source: (Sood, R., Vajdovich. P, 2013, p. 22)

3.4.2 Canine Malignant Mammary Gland Neoplasms with Advanced Clinical Staging Treated with Carboplatin and Cyclooxygenase Inhibitors:

The following experiment was carried out in 2012 by Geovanni Dantas Cassali, Gleidice Eunice LaValle, Cecilia Bonolo De Campos and Angelice Cavalheiro Bertagnolli in the Laboratory of Comparative Pathology, Department of General Pathology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil; Fepagro Animal Health, Institute of Veterinary Research Desidério Finamor, Eldorado do Sul, RS, Brazil. (Dantas Cassali et al., 2012)

The aim of the experiment was to compare the overall survival time and prognosis of female dogs with advanced mammary gland cancer when treated with different treatment protocols for surgery, chemotherapy, and different cyclooxygenase inhibitors.

During the experiment twenty-nine female dogs containing advanced mammary gland tumours were present. The patients were divided into four different groups as follows ; Group 1 consisted of seven dogs which would receive surgical treatment alone, Group 2 consisted of eight dogs which would undergo surgical excision of their tumours and then were subjected to treatment with carboplatin at a dose for 300mg/m2 for 21 day intervals and a total of three cycles, Group 3 consisted of five dogs which underwent surgical excision and the same treatment cycle of carboplatin as Group 2, however they were then treated with oral Piroxicam at a dose 0.3mg/kg every twenty four hours for six months. Group 4 consisted of nine dogs which also received the same treatment as Group 2. However, after the treatment they were given Previcox(firocoxib) orally at a dose of 5mg/kg every 24 hours for 6 months (as opposed to piroxicam in Group 3).

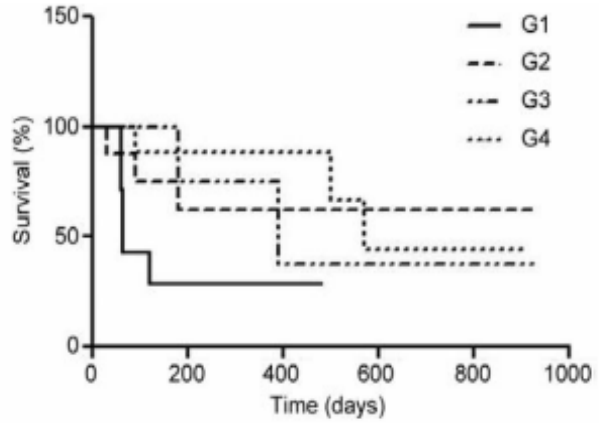
After surgery had been completed, the samples collected were processed by histopathological techniques to establish a histopathological diagnosis. Immunohistochemistry analysis was performed to determine the presence of COX-2 within the cells and COX-2 expression was analysed via cytoplasmic staining. Survival analysis was performed over a period of five years with clinical analysis follow ups taking place between 2005-2010 with check-ups being performed every 2 months. During the analysis chest x-rays were performed to evaluate any disease evolution particularly paying attention to recurrence or metastasis (particularly in the pulmonary region). Lymph node metastasis was confirmed by histopathological analysis. Complete biochemistry analysis and hemograms were performed to evaluate the side effects of chemotherapy performed. The survival time for this experiment was defined as the period between surgical excision of the tumour till the time of death when caused by the disease.

Out of the 29 tumours which were analysed, seven were carcinomas in mixed tumours, six solid carcinomas, five carcinosarcoma, five tubulopapilary carcinomas, one anaplastic carcinoma, one pleomorphic lobular carcinoma, one squamous cell carcinoma and three

micropapillary carcinomas. 24 of the 29 animals present metastasis in only one lymph node, five others in more than one lymph node and four presented metastasis in the lungs. A low COX-2 score was present in 41.4% of the patients examined and a high COX-2 score was present in 58.6% of the patients present. Patients with a high COX-2 score had a median survival time of 390 days while those with a low COX-2 score did not reach the median survival time.

The **Figure 6** below shows the relative survival time of all the different groups utilised within this experiment.

Figure 6 – Survival Time of Experiment Groups



Source: (Dantas Cassali et al., 2012)

As can be seen in **Figure 6** above, the overall survival time of each group of animals receiving treatment, that being Groups 2-4, was significantly increased when compared to Group 1 where the only form of treatment was surgical excision. Notably both Group 3 and Group 4 had the highest median survival time, 390 days for Group 3 and 570 days for Group 4. Both of these groups received NSAIDs with Group 4 receiving our targeted firocoxib and having the highest survival time. It must be noted that during the experiment one animal died due acute hemorrhagic gastro enteritis which was a side effect cause by the NSAID piroxicam within Group 3, however no side effects of firocoxib were presented.

This study was successful in showing how the utilization of firocoxib together with cisplatin can be vital for prolonging the survival time of animals with mammary gland cancer while producing relatively no or mild side effects as opposed to other NSAIDs or no chemotherapy at all.

3.4.3 Adjuvant Therapy for Highly Malignant Canine Mammary Tumours: Cox-2 Inhibitor Versus Chemotherapy: A Case–Control Prospective Study

The following study was carried out at the Veterinary Teaching Hospital (University Complutense, Madrid) (VTH-UCM) by C. Arenas, L. Peña, J. L. Granados-Soler, M. D. Pérez-Alenza and took place between 2008 and 2011. (Arenas, Peña, Granados-Soler and Pérez-Alenza, 2016)

The aim of the study was to observe the effects of adjuvant therapy, in this case the utilisation of mitoxantrone and firocoxib, on the overall survival time as well as the disease-free survival time when compared to no treatment after surgical resection of high-grade mammary gland tumours.

During the tests 28 female dogs were presented which contained at least 1 high grade malignancy mammary gland tumour (clinically stage 4 of histologically grade 3). The mean age of the subjects was 10.8 years. 7 of the 28 subjects had been neutered and 3 dogs had been giving hormonal treatment to prevent oestrous and 6 dogs had an irregular oestrous cycle. Prior to the beginning of the treatment all the necessary parameters were noted including the CBC, serum biochemistry and thoracic radiography. Surgical resection of the mammary glands as well as the lymph nodes affected by the tumour was then performed and histological investigations were then performed on the specimens collected. The results collected were analysed using a statistical software package and the descriptive statistics performed included age, breed, reproductive status, previous oestrous, regularity of oestrous, preventative hormonal treatments, number of malignant tumours, tumour size, metastasis and adherence to any other tissues, histological diagnosis and malignancy grade as well as the expression of the Ki-67 cell proliferation index (a protein which increases upon cell replication) and COX-2 immunohistochemistry staining.

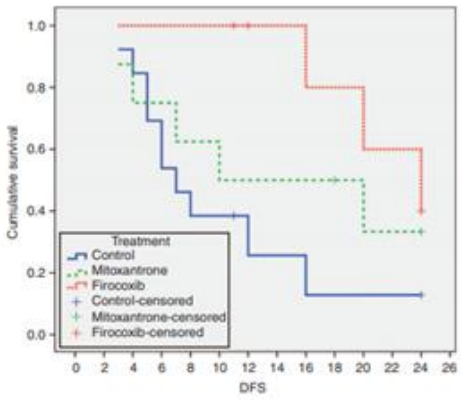
The patients were divided into 3 groups. Group A consisted of dogs which had received five doses of mitoxantrone at a dose of 5.5mg/m2 IV every 21 days for 24 months, Group B received firocoxib at a dose of 5mg/kg body weight orally every day for 24 months and Group C was the control group which had received no adjuvant therapy. Dogs in Group A had a re-evaluation performed every 21 days in the first four months and then once every 3 months after that. Dogs in Group B and C had been re-evaluated 1 month after treatment and then once every 3 months after. The re-evaluations consisted of a physical exam, CBC, and serum biochemistry as well as thoracic radiography. Some examinations were not performed for certain groups at certain times. The re-examinations were performed for 2 years or until death of the subject.

In Group A one dog had developed neutropenia, diarrhoea, and anorexia to a severe enough extent that the therapy had to be stopped and symptomatic therapy applied. The same side effects but to a much lesser extent were present in other patients also within this group. In Group B two dogs had developed Grade 1 anorexia and creatinine and urea levels had been increased and a renal diet had to be applied. The treatment was effective and the application of firocoxib did not have to be stopped. Therefore, as suspected the severity and presence of side-effects when utilising firocoxib is relatively low compared to its counterparts.

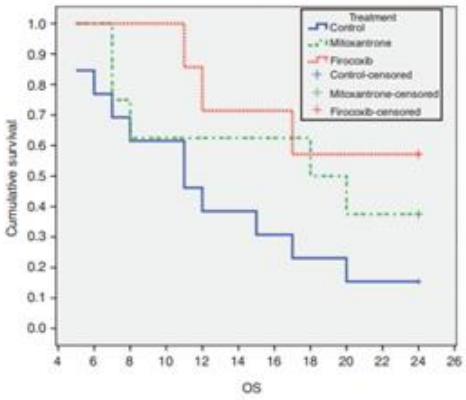
In Group A, local reoccurrence had developed in 3 dogs and pulmonary metastasis had developed in 2 dogs, only 3 of the original 8 had survived the full 24 months. 1 dog had died due to congestive heart failure. In Group B, 2 dogs developed local reoccurrence and 1 dog developed pulmonary metastasis and 4 of the original 7 had survived the full 24 months. One had died due to primary intestinal neoplasia, and one had died due to age related factors. In Group C 1 dog had developed local reoccurrence and 9 had developed pulmonary metastasis. It must be noted that all dogs in this group which developed distant metastasis had to be euthanised. Only 2 of the original 13 dogs in this group had survived by the end of the study, 1 had died due to diabetes mellitus and one other due to age related factors. All other unaccounted-for deaths were due to lesions directly or related to the mammary gland tumours.

The disease-free survival times in Group A was 14.3 ± 8.6 months and the overall survival time was for this group was 16.5 ± 2.6 months. The disease-free survival time in Group B was 21.6 ± 6 months and the overall survival time was 19.4 ± 2.1 months. The disease-free survival time of Group C was 10.1 ± 5.8 months, and the overall survival time was 12.7 ± 1.8 months. Chart 2 below represent the information of this paragraph.

Figure 7 – Cumulative Survival Time



Kaplan–Meier disease-free survival (DFS) curve of dogs included in the study. Dogs without tumour recurrence were censored



Kaplan–Meier overall survival (OS) curve of dogs included in the study. Dogs alive at the completion of the study were censored

Source: (Arenas, Peña, Granados-Soler and Pérez-Alenza, 2016)

No significance was seen in the disease-free survival time and the overall survival time between the groups receiving mitoxantrone and firocoxib (however those receiving firocoxib did express a slightly higher survival time). Nonetheless, the disease-free survival time and the overall survival time was significantly increased when comparing the group who had received firocoxib against the group who had not received any adjuvant therapy. The disease-free survival time of dogs which contained clinical stage 4 tumours was shorter when compared with those which had clinical stages 1, 2 or 3 tumours.

From this study it can be concluded that the utilization of adjuvant therapy, in post-op treatment for canine mammary gland tumours can be of significant value when trying to increase the overall survival time and reduce metastasis of the tumour and if possible, the utilization of firocoxib may be used for an overall better prognosis and reduction of side effects.

3.4.4 Clinical and Immunohistochemical Evaluations of Breast Tumours in Bitches Submitted to Treatment with Inhibitor of Cyclooxygenase 2 (Firocoxib)

The experiment was carried out at the Veterinary Center of Veterinary Medicine Course at PUC Minas University Campus Wells of Caldas by Luiz Henrique de Arauja Machado, Flavio Elston, Lilian Baretto Elston, Matthew Jose Sudano, Carlo Eduardo Fonesca and Fabiola Soares Zahn, which was officially accepted on 1st November 2014. (Machado et al, 2014)

The aim of the following experiment was to examine the expression of COX-2 within ductal mammary carcinomas of dogs and evaluate the effects that firocoxib would have on the expression of COX-2 within these tumours; as well as determine the effectivity of the use of firocoxib utilizing clinical and laboratory paraments for bitches with breast tumours.

During the experimentation 19 dogs were submitted for testing and were divided into two groups, one group contained 9 animals which did not receive any treatment and acted as the control group while the other group consisted of the remaining 10 animals were treated with firocoxib. All animals underwent a physical and clinical examination prior to the start of the experiment which consisted of tumour measurements via caliper as well as mapping, chest radiographs, blood, and biochemistry analysis. Tumour samples were then collected via incisional biopsy. After the biopsy sampling, the animals within the treatment group received a dosage of 5mg/kg body weight per day of firocoxib for seven days. After these seven days both animal groups were subjected to surgery, excisional biopsy and blood collection. The tumour samples collected were classified either as carcinoma, benign mixed tumour or carcinoma in mixed tumour. Immunohistochemistry was performed on sections of the samples to determine COX-2 expression and were classified as either having low, moderate or intense expression.

Analysis of variance test had been performed from the data collected before and after treatment and when statistical variance was determined a further Tukey test was used to evaluate the interaction between the factors. The McNamar test was used to compare the

proportional difference of each type of tumour classification before and after the 7-day treatment and a Students T test was used to compare the effects of treatment before and after 7 days with the parameters evaluated in each group. The data utilized and results obtained from the experiment are present within Figures 8 & 9:

Figure 8 – Control vs Treatment Group

Control Group					Treated Group				
Animal	Age (Years)	Number of Nodes	*CCT	**CHT	Animal	Age (Years)	Number of Nodes	*CCT	**CHT
1t	9	3	1	1	1	9	5	3	1
2t	5	2	3	3	2	9	6	3	1
3t	8	1	1	1	3	5	1	2	2
4t	10	7	2	1	4	10	1	3	3
5t	8	1	1	1	5	11	3	1	1
6t	13	3	2	2	6	4	3	3	1
7t	11	3	3	1	7	9	6	1	1
8t	11	4	3	1	8	7	3	1	1
9t	7	4	3	2	9	12	4	1	1
10t	12	2	1	1	--	--	--	--	--

(Data on age, Number of nodules (NN), cytological tumour classification (CCT) and histological classification of the tumour (CHT))

Figure 9 – Statistical Results

	VMN (cm)	COX2 (0-3)	UR (mg/dL)	CRE (mg/dL)	AST (UL/L)	ALT (UL/L)	
Group	D0	D7	D0	D7	D0	D7	
Control	Mean	27.54	28.69	0.67a	1.11b	44.22	
n = 9	SD	27.53	28.49	0,50	0.78	11.14	
Treated	Mean	12.51	14.00	1.00	0.70	34.6a	
n = 10	SD	11.62	14.71	0.67	0.95	14.00	

PT (g/dL)										
Group	VMN (cm)	D0	D7	D0	D7	D0	D7	D0	D7	
Control	Mean	40.44	0.90	0.95	13.55	13.44	26.44A.a	22.22 b	6.87	6.69
n = 9	SD	4.75	0.245	0.3087	3.71	3.1667	9.66	10.53	1.28	1.51
Treated	Mean	46.80 b	0.95	1.03	15.5	17.20	16.10B	20.30	6.72a	5.92b
n = 10	SD	18.28	0.52	0.51	6.43	12.38	7.79	9.52	1.23	1.02
SD - Standard Deviation										
VMN - Volume Parameters of Largest Nodules										
UR - Serum Levels of Urea										
CRE - Creatinine										
DO - Day 0										
D7 - Day 7										

Source: (Machado et al, 2014)

As can be seen above, ALT levels were significantly reduced from day 0 to day 7 within the control group. In the treated group no such variation was observed, the total protein amount was significantly reduced between day 0 and day in the treated group, Serum urea was significantly increased during the two test times within the treated group, expression of COX-2 was significantly increased in the control group between the two test times. This change was not verified in the treated group. All other paraments measured showed no significant statistical variation between times of testing within either group.

From the results obtained we can conclude that firocoxib was effective in performing its proprietary role in reducing the amount of COX-2 enzyme and therefore the harmful effects resulting from the subsequent inflammation. However, it must be noted that no significant decrease in tumour size was measured; but also no metastasis was present. The increase in serum urea levels in the treated group may be indictive of acute kidney injury which is suspected to be due to the inhibition of renal production and systemic PGI-2 which decreases sodium excretion. Therefore, caution and constant monitoring could be suggested when treating animals who are subject to renal complications with firocoxib. The decrease in plasma protein levels is suspected to be due to gastrointestinal bleeding however no such conclusion could be made as no bleeding was observed during the experimentation.

4.0 Summary and Conclusion

After having read and compiled the data present from several studies for both firocoxib and chlorambucil a positive conclusion may be drawn from their use for not only mammary gland cancer but also for several other forms of cancer. While the data collected is far from 100% conclusive with more experimentation required; primarily regarding tests utilizing larger groups to account for several variables that may be presented when these chemotherapeutic agents are used in practice. This will potentially lead to the full approval of these drugs by the FDA and WHO which will allow for full confidence when using such agents in said practice.

Primarily tests were first carried out using laboratory animals such as mice and rabbits to provide a general idea of the safety of use of such agents and once this invaluable information was available, further research into other species was prompted. Since canine mammary gland cancer serves as a model for human mammary gland cancer, this species was of primary concern for researchers as it would provide information that is applicable in human medicine, while also being an important factor when receiving funding for such studies.

Chlorambucil is one of several alkylating agents, however the metronomic application of this agent provides us with safer alternatives to classically used alkylating agents such as cyclophosphamide due to having lessened severity or absence of certain side effects such as urinary cystitis. The search for chemotherapeutic agents with little to no side effects is of a higher concern for cancer treatment more so than any other diseases due to the sensitivity of such a situation. The further study and use of Chlorambucil in practice may eventually lead to the development of other alkylating derivates which may hopefully and eventually provide the desired anti-cancer effects without any of the undesired side-effects.

Much like chlorambucil, firocoxib provides a valuable alternative to traditionally used chemotherapeutic agents. In this case the main side effects associated with the use of traditional NSAIDS, namely the gastrointestinal disorders are for the most part avoided by being having specific interactions with the COX-2 enzyme. This interaction is of particular interest when trying to find alternative chemotherapeutic agents with little to no side effects

as it provides important insight to achieve such results. Agents which interact with specific enzymes of certain biological processes or act within certain cells only shall be required to have the desired effect only within the carcinogenic cells thus sparing healthy cells and avoiding the consequences of the side effects that would have otherwise been present.

While many studies have been carried out on the effects of chlorambucil and firocoxib, the information is not totally complete This is especially so, with regards to the seemingly positive potential of applying these agents metronomically. Moreover, there is also a lack in up to date and recent studies done of both agents with regards to cancer. While new studies utilizing much larger test groups of canines can provide us with valuable information for the treatment of cancer - not only from a veterinary standpoint but also from a human medicine perspective; studies may be carried out on other species which seem to have not been yet tested. The data obtained from these experiments may provide us with valuable information on other potential uses of the agents for other disease such as the use of firocoxib for musculoskeletal disease. There is also a lack in information regarding the use of these agents with a wide variety of other pharmaceutical agents which when used synergistically, may possibly produce substantial positive effects, or provide us with further information regarding their safe application.

Moreover, it seems to be important to perform more clinical and model experiments which help to understand the mechanism of action using these two compounds as chlorambucil and firocoxib.

Despite this lack of research, a recent and important initiative has been taken-up to address the situation. Recently an international oncology group called VOAB (Veterinary Oncology Advisory Board) has been established. This involves veterinarians from Spain, Portugal, England, Sweden, Poland, The United States, Japan, France, Germany and Hungary. They have designed an international, multicentric study for the evaluation of clinical efficacy of chlorambucil/firocoxib metronomic therapy for dogs with mammary gland tumours. The inclusion criteria include dogs with grade III and/or Stage IV cases, as the less advanced cases do not need adjuvant chemotherapy (Peña L, Andrés PJD, Clemente M, Cuesta P, Pérez-Alenza MD. Prognostic Value of Histological Grading in Noninflammatory Canine

Mammary Carcinomas in a Prospective Study with Two-Year Follow-Up: Relationship with Clinical and Histological Characteristics. Veterinary Pathology. 2013;50(1):94-105. doi:10.1177/0300985812447830). The project started in 2015. The patient collection is currently in its final stages. After finalizing the results, this group will be able to provide valuable information about the efficacy of chlorambucil/firocoxib metronomic chemotherapy in canine mammary gland tumours.

Such international alliances do indeed provide an important step in advancing our understanding and knowledge effects of chlorambucil and firocoxib on mammary gland cancer cells.

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