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Journey from the Bedside to the Bench and Back

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GENETIC VARIABILITY IN GENES RESPONSIBLE FOR METABOLISM OF CHEMOTHERAPEUTIC DRUGS: JOURNEY FROM THE BEDSIDE TO THE BENCH AND BACK

By

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ABSTRACT

GENETIC VARIABILITY IN GENES RESPONSIBLE FOR METABOLISM OF CHEMOTHERAPEUTIC DRUGS:
JOURNEY FROM THE BEDSIDE TO THE BENCH AND BACK

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Dacarbazine is a chemotherapeutic that requires activation by cytochrome-P450 enzymes, mainly from CYP1A1, CYP1A2 and CYP2E1. While these genes are known to be polymorphic in humans, no study to date has investigated polymorphisms at this locus in client-owned dogs. We hypothesize based on clinical data from to studies conducted in dogs treated with a dacarbazine chemotherapy protocol for their cancer that genetic variation exists in the coding region of both these genes. The canine CYP1A1, CYP1A2, CYP2E1, and CYP2B11 (homologue to CYP2B6) genomic sequences were identified using the UCSC Genome Browser. Intronic primers, flanking individual exons were designed using Primer3. Amplification of all exons was carried out by PCR. Genomic DNA from 20 dogs (10 breeds, 2 dogs / breed) from our lab's genomic DNA bank was used as template. Direct sequencing was performed and the results analyzed. RFLP assay was designed for each polymorphism identified by sequencing and were used to evaluate polymorphic sites in dogs of 10 different breeds (10 dogs /

breed). The potential effect of each polymorphism to the protein function was estimated using PolyPhen. In CYP1A1 a total of 6 SNPs were identified: three SNPs in exon 1, and three SNPs in exon 6. One SNP was a synonymous change (c.1362T>A), while five were nonsynonymous (c.53C>G → p.A18G; c.146G>T → p.W49L; c.345C>G → p.F115L; c.C1305C>A → p.F435L; and c.1561T>C → p.S521P). In CYP1A2, a total of 6 SNPs were identified. SNPs have been already described (c.1117C>T; c.1173C>G; c.1299C>T; and c.1303G>A), while 2 novel SNPs were identified (c.1165A>G; and c.1451A>G). Of the novel SNPs, one is a synonymous change (1451A>G), and one is nonsynonymous (c.1165A>G → p.N389D). A potential InDel was identified at the 6^{th} exon in 7/13 Siberian huskies initially assayed. We identified 75 homozygotes for the wild type, and 199 dogs that appeared heterozygotes for the duplication. In CYP2E1, a total of 2 SNPs in the coding region and 2 intronic SNPs were identified. Two exonic SNPs are nonsynonymous (c.85C>T → p.R29C; c.1453T>C → p.Y485H). The two intronic SNPs are located at the 5^{th} (IVS5+5G>A), and the 6^{th} intron, (IVS6+27C>T). In CYP2B11 a total of 3 SNPs were identified. Two SNPs were synonymous (c123G>A; c966G>A) and 1 was nonsynonymous (c220C>T → p.R74C).

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CHAPTER 1

INTRODUCTION

'Cytochrome P450' was proposed as a temporary name for a class of common enzymes characterized as having a colored pigment in the cell, and a 450 nm Soret peak when reduced and bound with carbon monoxide.(Omura and Sato 1962; Bachschmid, Schildknecht et al. 2005) The common thread of all CYP enzymes is a conserved peptide motif, Phe-X(6-9)-Cys-X-Gly (where X denotes any amino acid), near the C terminus. The cysteine binds octahedral heme iron in the fifth position and participates in the transfer of one atom of oxygen into the substrate, which is located in the nearby substrate binding pocket. Formation of the oxygenated product is then followed by its release; the product can be so unstable that within milliseconds it becomes a stable (poorly reactive) hydroxylated compound, or the product can be a relatively unstable reactive oxygen metabolite (ROM) capable of intracellular covalent binding.(Nebert and Gonzalez 1987)

All mammals have 18 *CYP* gene families (Appendix), with considerable variation in the number of members in many of the subfamilies of the *CYP2, CYP3* and *CYP4* families. For enzymes in the *CYP2, CYP3* and *CYP4* gene families, the

extrapolation of a rodent CYP gene to a human *CYP* gene is rarely possible owing to species differences in metabolism and the fact that human and rodent orthologs usually cannot be determined.(Nelson, Zeldin et al. 2004) Enzymes from the *CYP1* gene family and families higher than *CYP4* have necessary, distinct and separate functions.(Nebert 1991; Nebert 2000) However, virtually all CYPs are presumed to participate in many crucial subcellular pathways. As a result, genetic variation in these *CYP* genes may contribute to various clinically relevant diseases.(Nebert and Russell 2002)

Enzymes in the *CYP1, CYP2, CYP3* and *CYP4* gene families show some redundancy and overlapping substrate specificity; these CYP enzymes are most directly related to the development of cancer as a result of environmental carcinogens. Enzymes in other CYP gene families control the levels of sex steroids, corticosteroids, cholesterol, bile acids and morphogens such as retinoic acid; because these endogenous substrates are sometimes associated with tumor promotion, it might be concluded that these CYP enzymes participate indirectly in tumorigenesis.

Many allelic variants of human *CYP1, CYP2, CYP3* and *CYP4* genes exist, resulting in potential metabolic polymorphisms. A database of these *CYP* allelic variants is

maintained on the Human Cytochrome P450 Allele Nomenclature Committee web site, which uses a consistent classification system: the consensus (reference, or normal) *1 allele is responsible for the efficient metabolism phenotype. Variant alleles can sometimes cause an intermediate-metabolism or a poor-metabolism phenotype, the latter of which results in low (or no) enzyme activity.(Ingelman-Sundberg 2004; Nelson, Zeldin et al. 2004) Occasionally, as many as 12 additional copies of the same functional CYP gene can occur in tandem, with these variants believed to be responsible for the ultrahigh-metabolism phenotype.(Johansson, Lundqvist et al. 1993)

FUNCTIONAL ASPECTS OF CYP ENZYMES

As first proposed in 1949 by R.T. Williams, the metabolism of drugs and environmental pollutants can be divided into phase I (functionalization) and phase II (conjugation) reactions.(Williams 1949) CYP enzymes comprise 70-80% of all phase I xenobiotic metabolizing enzymes (XME).(Evans and Relling 1999) Most phase I XMEs are capable of both detoxication and metabolic activation. The metabolic activation of procarcinogens often leads to the formation of electrophilic intermediates.

Conjugations by phase II XMEs occur with moieties such as glucuronide, mercapturic acid, acetate, methyl, sulphate, glycine, glutamine, thiocyanate and glucoside, producing a very hydrophilic product that can be readily excreted, therefore completing the detoxication cycle.(Williams 1971) Although the combined effects of phase I and phase II XMEs usually result in the detoxication of foreign chemicals, ROMs, such as benzo[a]pyrene (BaP) trans-7,8-dihydrodiol-9,10-epoxide and the benzene o- and p-quinones, readily bind covalently to DNA and proteins, and are strong mutagens and carcinogens by virtue of their highly reactive electrophilic groups.

Very hydrophobic chemicals, such as BaP or benzene, enter the cell passively, and require no enzyme or other energy-requiring process. Some environmental toxicants enter the cell through endogenous receptors, membrane-bound transporters or ion channels. A fundamental rule of toxicology is that all XMEs, XME receptors and xenobiotic related transporters (XRT) have evolved first for endogenous functions; most xenobiotics then simply 'hitch-hike' these endogenous systems to enter the organism and wreak havoc.(Nebert and Dalton 2006)

Some XMEs such as CYPs, are always membrane-bound, predominantly in the endoplasmic reticulum, mitochondria and, occasionally, the plasma membrane. A few XMEs such as certain oxidases, hydroxylases and reductases, are only found in the cytoplasm. However, most XMEs such as epoxide hydrolases, glutathione S-transferases and sulphotransferases have both cytosolic and membrane-bound forms. XMEs can also be intranuclear, but this field has been largely unexplored.(Wada, Kang et al. 2008)

Hydrophobic chemicals are presumably attracted to membranes, in which most phase I XMEs reside. Certain XMEs show substrate-specific binding, whereas many others have greater flexibility, with overlapping substrate specificity. Evidence indicates that drug metabolism occurs to some

degree in virtually all eukaryotic cells and in many prokaryotes. Therefore, each XME gene shows a high level of time-specific, organ-specific, tissue-specific and cell-type-specific expression.(Nebert and Vesell 2006)

GENETIC VARIABILITY OF THE CYTOCHROME P450 AND MEDICAL TREATMENT OF CANCER IN DOGS

Cancer is the leading cause of death in older animals. In an autopsy series of 2000 dogs, 23% of all dogs, regardless of age, and 45% of dogs over 10 years old, were found to have died of cancer.(Vail and MacEwen 2000) Estimates of age-adjusted overall cancer incidence rates per 100,000 individuals/years at risk range from 243 to 381. These rates are comparable to those reported by the National Cancer Institute SEER program for human beings.(Hansen and Khanna 2004) Companion animal owners, motivated by the desire to prolong the quality of their animals' lives, frequently seek out specialized pet care and treatment provided by veterinary oncologists.

Great efforts may be undertaken to extend life expectancy and provide tumor control in canine cancer patients with disseminated disease. However, chemotherapy is used largely in a palliative setting in veterinary oncology, since quality of life appears to be a more desirable goal than absolute tumor response. Most dogs tolerate multidrug chemotherapeutic protocols well, but some patients experience serious or lethal adverse effects despite the standardized drug dosing and scheduling.(Mellanby, Herrtage

et al. 2003) The occurrence of atypical, severe adverse reactions not only decreases the quality of life of the patient, but also appears to negatively affect overall survival.(Ahaus, Couto et al. 2000)

Chemotherapy is administered in both humans and dogs based on the assumption that each individual will metabolize drugs with the same efficiency. It is evident in human oncology practice that significant variation in adverse effects exists even when factors such as diet, co-administration of other drugs, and individual differences in liver and kidney function are accounted for. This leaves the potential for genetic variation, as an explanation of differing drug toxicities between individuals. Most anticancer chemotherapeutic drugs have a very narrow therapeutic window; thus, avoiding underdosing or overdosing becomes critical.

Many drugs used in veterinary oncology are substrates for phase I or phase II metabolizing enzymes. Cytochrome P450 (CYP) superfamily enzymes are critical in the phase I metabolism of drugs and xenobiotics, catalyzing mostly oxidative reactions. Many cytotoxic chemotherapeutic drugs, such as dacarbazine, cyclophosphamide, ifosfamide, paclitaxel, docetaxel, and vincristine, are dependent upon

CYP-catalyzed activation to exert their tumoricial effect.(McFadyen, Melvin et al. 2004)

Dacarbazine (DTIC) is a chemotherapeutic agent whose mechanism of action is not clearly understood. Three proposed mechanisms of action include: inhibition of DNA synthesis; action as alkylating agent; and interaction with cellular -SH groups.(Loo, Housholder et al. 1976) Dacarbazine is used in veterinary oncology for the treatment of refractory lymphoma, melanoma, and sarcoma, despite the lack of adequate pharmacokinetic studies in the cancer-bearing dog.(Loo, Tanner et al. 1968) The administration of dacarbazine requires slow IV infusion over 8 hours, or daily slow IV bolus injections over 5 days, every 3 weeks.(Gray, Raulston et al. 1984; Ahaus, Couto et al. 2000)

Dacarbazine is a prodrug that undergoes conversion to the linear triazine 3-methyl-(triazen-1-yl) imidazole-4-carboxamide (MTIC). MTIC subsequently undergoes spontaneous chemical conversion to become 5-amino-imidazole-4-carboxamide (AIC) and methyldiazonium ion, the putative active form of the cytotoxin.(Tsang, Quarterman et al. 1991) The methyldiazonium ion is the nucleophilic molecule that interacts with DNA and forms lethal methyl adducts at the O^6-guanine position. The activation process in humans is

believed to be dependent mainly on CYP1A2, CYP2E1, and CYP1A1.(Reid, Kuffel et al. 1999)

The CYP enzymes form a ubiquitous family of heme proteins involved in xenobiotic metabolism, biosynthesis of steroids, lipids, vitamins, and natural products. These enzymes catalyze a variety of reactions including hydroxylations, epoxidations, N- or O-dealkylations, and Baeyer-Villiger oxidations.(Chefson and Auclair 2006) The P450 superfamily is divided into families (i.e. CYP1, CYP2, CYP3, CYP4) where the primary protein structure is identical by more than 40% among family members.(Zuber, Anzenbacherova et al. 2002) Each family is divided in turn into subfamilies (labeled with letters, i.e. A, B, C, D, E) where the primary structure is at least 55% identical. The site of CYP action can differ, beginning with the organs where xenobiotics enter the body such as liver and the gastrointestinal tract, lung, and nasal mucosa, extending to excretory organs and tissues as the kidney, and tissues such as skin or blood cells.(Krishna and Klotz 1994)

The CYP enzymes have been shown to have multiple alleles in humans. These alleles generally behave as major loci for metabolic effects. Knowledge of *CYP* allelic variants in human population is rapidly increasing, rendering these enzymes amendable as potential targets for

genetic screening, thereby allowing for individualized medical treatment. The variation in *CYP* genes may cause absence of enzyme, diminished enzyme expression, enzyme with altered substrate specificity or increased enzyme expression. Based on the composition of the alleles, the affected individuals can be divided into four clinically relevant phenotypes: poor metabolizers having two nonfunctional genes; intermediate metabolizers being deficient in one allele; extensive metabolizers having two copies of normal genes; and ultrarapid metabolizers having three or more functional active gene copies.(Rodriguez-Antona and Ingelman-Sundberg 2006)

Not much data are available for specific *CYP* expression and activity in the dog. While over 60 *CYP* genes are believed to exist, only nine have been even partially characterized in the dog.(Graves, Elhag et al. 1990; Uchida, Komori et al. 1990; Ciaccio, Graves et al. 1991; Sakamoto, Kirita et al. 1995; Fraser, Feyereisen et al. 1997; Blaisdell, Goldstein et al. 1998; Lankford, Bai et al. 2000) Despite the significant lag in the canine *CYP* characterization, it appears that large interspecies differences might exist for many CYP enzymes.(Walton, Dorne et al. 2001) Additionally, human CYP variants corresponding to poor and extensive metabolizers have been demonstrated in

the dog, with the most notable example being the presence of *CYP1A2* mutants in research beagle colonies.(Mise, Hashizume et al. 2004; Tenmizu, Endo et al. 2004) Our research focuses on further characterization of this important family, as will be described below.

Early studies of a few specific genes have illustrated the importance of pharmacogenetics for the clinical well being of canine patients. The toxic effects of ivermectin on some dogs, particularly Collies, were discovered to be due to a mutation in the *MDR1* gene.(Neff, Robertson et al. 2004) Once this mutation was discovered, it was found to be present at a lower frequency in several other herding breeds.(Neff, Robertson et al. 2004; Geyer, Doring et al. 2005; Mealey and Meurs 2008) Genetic testing of dogs in these breeds is now commercially available, so that clinicians can prevent harm to dogs at risk, by avoiding the toxic effect of ivermectin. Interestingly, metabolism of specific chemotherapeutic agents including vincristine are also dependent on the *MDR1* gene.(Mealey, Northrup et al. 2003; Culmsee, Gruber et al. 2004; Mealey, Fidel et al. 2008) A second example of the importance of identifying the genetic basis of adverse drug reactions is the induction of malignant hyperthermia in some dogs, particularly greyhounds, by the use of certain anesthetic agents. A

mutation in the *RYR1* gene was found to be the culprit.(Roberts, Mickelson et al. 2001)

Most informative to our research, two groups of investigators reported a *CYP1A2* knockout mutation in colonies of research beagle dog that has been shown to have a dramatic impact on the variation of experimental pharmaceuticals.(Mise, Hashizume et al. 2004; Tenmizu, Endo et al. 2004; Tenmizu, Noguchi et al. 2006) Based upon the recognized importance of genetic variation in *CYP* genes for human drug metabolism, and the fact that some important anti-cancer drugs such as dacarbazine cause toxicity in some but not all dogs, we believe that it is quite likely that variation in canine *CYP* genes will be found to have an important impact on canine drug metabolism as well. Identifying these variations will lead to improved care for individual canine cancer patients by allowing more precise tailoring of drug selection and dosing for optimal efficacy while avoiding undesirable and potentially lethal toxicity.

In the human *CYP* gene family, the most penetrant genetic alterations are gene deletions, missense mutations, and mutations that create splicing defects and premature stop codons. With few exceptions mutations in the 5′- or 3′-untranslated regulatory regions do not appear to affect the *CYP* phenotype. Despite this, a large amount of literature

reports association studies linking such low-penetrance polymorphisms to the incidence of severe diseases, among them various types of cancer.(Agundez 2004; Ingelman-Sundberg 2004)

CYP1A1

Most of the known environmental carcinogens are chemically inert in themselves and require metabolic activation by cytochrome P450 (CYP) enzymes to more reactive metabolites in order to exhibit carcinogenicity in experimental animals and humans.(Conney 1982) Of the 17 families of human CYPs identified to date, the CYP1, 2, and 3 family members play major roles in the metabolic activation of a variety of environmental carcinogens. It has been suggested that CYP1A1 is responsible for the activation of most carcinogenic polycyclic aromatic hydrocarbons (PAHs) to epoxide intermediates, which are further converted to more reactive diol-epoxides through the activity of epoxide hydrolase.(Conney 1982; Shimada, Yamazaki et al. 1996)

The polycyclic aromatic hydrocarbon carcinogens are ubiquitously distributed in the environment and their carcinogenic potentials have been extensively studied in experimental animal models. Historically, CYP1A1 had been thought to be the sole enzyme responsible for the metabolic activation of most of the carcinogenic PAHs to reactive electrophiles in mice, rats, and rabbits. PAHs and polyhalogenated hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induce the expression of

several xenobiotic-metabolizing enzymes, including CYP1A1, through the arylhydrocarbon receptor (AhR). Increased synthesis of these enzymes, as well as genetic variations in the *CYP1A1* gene, may determine the different susceptibilities of individuals to carcinogenesis caused by PAHs.(Shimada and Fujii-Kuriyama 2004)

Expression of *CYP1A1*, which catalyzes the 2-hydroxylation of estradiol, is induced by numerous PAHs and aryl amines, as well as by TCDD.(Guengerich 1988; Whitlock 1999) This induction is mediated by the AhR and AhR nuclear translocator (ARNT).(Hankinson 1995; Whitlock 1999) The ligated-AhR/ARNT heterodimer interacts with the xenobiotic responsive element (XRE) located in the enhancer region of the target genes, and subsequently transcription of the target gene is enhanced. At least seven XREs have been identified in the promoter region of the human *CYP1A1* gene.(Fisher, Wu et al. 1990; Kubota, Sogawa et al. 1991; Whitlock 1999) Recently, in a study using cDNA microarray analysis, it was reported that the expression level of CYP1A1 mRNA in human breast cancer MCF-7 cells is stimulated by treatment with estradiol (Frasor, 2004).(Stossi, Barnett et al. 2004) However, the induction mechanism has not yet been clarified.(Tsuchiya, Nakajima et al. 2005)

For the human *CYP1A1* gene, several variant alleles, including *CYP1A1*1B* to *CYP1A1*11*, have been identified. Among them, *CYP1A1*2A* gives rise to an MspI restriction site in the 30-noncoding region at T3801C and *CYP1A1*2C* results to an amino acid substitution at codon Ile462Val. The sequence variations have been reported to significantly elevate the inducible enzymatic activity of CYP1A1 compared to the wild-type genotype. Some studies reported that *CYP1A1*2A* and **2C* variants increase the risk of breast cancer.(Ambrosone, Freudenheim et al. 1995; Taioli, Trachman et al. 1995) The CYP1A1*2C allele also appears to be associated with increased risk of prostate and ovarian cancer.(Murata, Shiraishi et al. 1998; Goodman, McDuffie et al. 2001) Endometrial cancer was also related to *CYP1A1* polymorphisms and a minor or negative association of cervical cancer with the *CYP1A1* genotype has been reported.(Esteller, Garcia et al. 1999; Longuemaux, Delomenie et al. 1999)

CYP1A2

The human cytochrome P450 enzyme CYP1A2 plays an important role in the metabolism of several clinically useful drugs. It is one of the major P450 enzymes and accounts for approximately 13% of the total content of this enzyme group in the human liver.(Shimada, Gillam et al. 1994) CYP1A2 mRNA content demonstrates an up to 40-fold variability between individuals, with corresponding variability of enzyme activity and drug metabolism.(Schweikl, Taylor et al. 1993; Potkin, Bera et al. 1994)

The CYP1A2 enzyme is constitutively expressed preferentially in liver.(Quattrochi and Tukey 1989) The basal expression of the human *CYP1A2* gene is regulated by transcriptional factors such as liver specific transcription factor 1 and upstream stimulatory factor.(Chung and Bresnick 1997; Pickwell, Shih et al. 2003) *CYP1A2* is also induced by PAHs, as well as TCDD via the AhR/ARNT complex.(Hankinson 2005) It has been reported that administration of estradiol increases the expression of CYP1A2 protein in the hamster testicles.(Hudson, Schulte et al. 2001) Therefore, estrogens may affect the expression level of *CYP1A2*. However, information about the exact mechanisms by which estrogen

regulates CYP1A2 activity is limited.(Tsuchiya, Nakajima et al. 2005)

In the human *CYP1A2* gene, variant alleles *CYP1A2*1B* through *CYP1A2*14* have been identified. It has been reported that enzyme activity is decreased in the *CYP1A2*7* variant, which causes a splicing defect and by *CYP1A2*11*, which causes an amino acid substitution.(Allorge, Chevalier et al. 2003; Murayama, Soyama et al. 2004) However, no conclusive evidence that CYP1A2 polymorphisms are associated with altered cancer risk has been published.

THE QUESTIONABLE UTILITY OF THE *CYP1* FAMILY GENES

The original 1940s naming scheme for cytochrome P450 enzymes emphasized their detoxication function, and implied that CYPs and other phase I XMEs were uniformly beneficial.(Williams 1949) During the late 1960s, experiments incubating benzo[a]pyrene (BaP) and microsomes with DNA or protein *in vitro* it was demonstrated that BaP reactive intermediates became covalently bound to DNA and protein.(Grover and Sims 1968) This discovery, identifying BaP DNA and protein adduct formation, demonstrated that, during CYP - mediated conversion from parent compound to excreted hydrophilic conjugated product, ROMs are formed that are capable of binding covalently to nucleic acids and proteins. The non-metabolized parent compound was, therefore, termed a procarcinogen, with the ultimate carcinogen appearing only after CYP metabolism.

Therefore, the debate as to whether some CYP enzymes are more harmful than beneficial still exists. In CYP knockout mouse experiments, no overt phenotype was described and these mice are able to reproduce normally.(Liang, Li et al. 1996; Buters, Sakai et al. 1999; Dalton, Dieter et al. 2000) However, the CYP1B1-/- mouse has increased protection against many different PAH - induced malignancies compared

with wild-type mice. Therefore, as predicted from earlier cell culture and in vitro studies, if CYP1B1 is not present to activate these xenobiotics, less toxicity is seen. The Ahr-/- mouse has lowered viability, fertility and failure to close the ductus venosus, which leads to impaired liver development.(Harstad, Guite et al. 2006) The Ahr-/- mouse lacks most basal and all inducible *CYP1* expression, and is resistant to several PAH - induced and dioxin-induced malignancies.(Fernandez-Salguero, Pineau et al. 1995) As anticipated from earlier cell culture and in vitro studies, decreased genotoxicity and mutagenesis is seen in the absence of AHR-mediated *CYP1* induction.(Nebert, Dalton et al. 2004)

However, the CYP1A2-/- mouse demonstrates paradoxical responses to carcinogen treatment. In cell culture and in vitro studies, the CYP1A2-mediated metabolic activation of the human carcinogen 4-aminobiphenyl (ABP) or the food mutagens 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) caused increased adduct formation and mutagenesis. Therefore, in a CYP1A2-null animal one would expect protection against ABP, IQ or PhIP. However, CYP1A2-/- mice treated topically with any of these three procarcinogens unexpectedly show increased adduct formation, methemoglobinemia and several

types of malignancies in target organs.(Shertzer, Clay et al. 2004)

The CYP1A1-/- knockout mouse also shows paradoxical effects. In cell culture and in vitro studies, the metabolic activation of BaP by CYP1A1 is known to increase BaP-DNA adduct formation and mutagenesis. Therefore, the CYP1A1-null animal should be protected from BaP; however, daily oral BaP treatment of the CYP1A1-/- mice rapidly resulted in the death of these animals. Pharmacokinetic studies showed that, as expected, BaP accumulates to higher levels in CYP1A1-/- mice than wild-type mice. In all tissues examined, levels of BaP-intermediates to DNA adducts are much higher in CYP1A1-/- mice than in wild-type mice. Furthermore, immunosuppression occurs in CYP1A1-/- mice but not in wild-type mice.(Uno, Dalton et al. 2004)

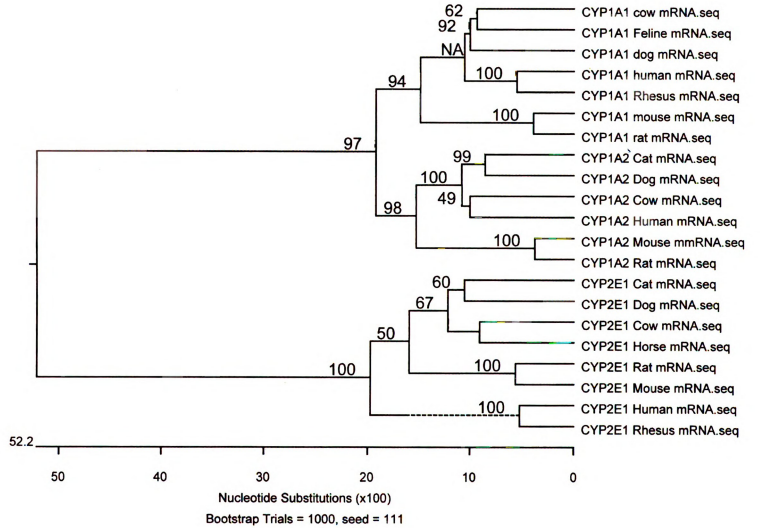
CYP2E1

CYP2E1, expressed in the liver and many other tissues, is an important enzyme from the standpoint of metabolism of clinical medications and in toxicology with regard to the metabolism of xenobiotics. This CYP isoform metabolizes a number of small, hydrophobic compounds (molecular weight <100). The enzyme substrates include endogenous compounds such as ketones and fatty acids, and industrial solvents such as chloroform.(Koop and Casazza 1985; Imai 1988; Laethem, Balazy et al. 1993; Nakajima, Elovaara et al. 1995) Many therapeutic agents, such as acetaminophen, ethanol, and volatile anesthetics, including isoflurane and sevoflurane, have been shown to be substrates for CYP2E1.(Coon, Koop et al. 1983; Morgan, Koop et al. 1983) In addition, CYP2E1 also plays a significant role in the metabolic activation of procarcinogens, including benzene and nitrosamines.(Koop, Laethem et al. 1989; Yamazaki, Oda et al. 1992) Thus, CYP2E1 might be an important determinant of human susceptibility to toxicity and carcinogenicity of industrial and environmental chemicals.

However, polymorphisms affecting *CYP2E1* expression or activity have not been described, possibly because of a high level of evolutionary conservation due to the critical role

of this enzyme in gluconeogenesis during conditions of starvation. By contrast, induction of the enzyme by chemicals such as, alcohol might provide a more important component of interindividual susceptibility to cancer in reactions mediated by CYP2E1. The CYP2E subfamily is unique in the CYP2 family. It has been shown from studies of numerous species that the CYP2E subfamily has only a single isoform in most mammals, with the exception of rabbits. Other subfamilies belonging to the CYP2 family include multiple isoforms.(Khani, Porter et al. 1988)

Figure 1. Phylogenetic tree of selected CYP genes



CHAPTER 2

Treatment with Doxorubicin, Dacarbazine, and Vincristine (DAV) for Advanced Stage Hemangiosarcoma in Dogs

INTRODUCTION

Hemangiosarcoma (HSA) is a malignant tumor that arises from endothelial cells. The disease is fairly common in the dog, while it is extremely rare in humans.(Priester 1976) German
Shepherd Dogs, Golden Retrievers, Labrador Retrievers, and Schnauzers appear to be predisposed.(Brown 1985; Clifford, Mackin et al. 2000) Canine HSA is typically a highly malignant tumor, frequently metastasizing to distant organs such as liver, lungs, heart, skin, and central nervous system (CNS).(Brown, Patnaik et al. 1985) Many patients are presented with internal bleeding due to tumor rupture or disseminated intravascular coagulation (DIC) and are subsequently diagnosed with the disease.(Legendre and Krehbiel 1977; Ng and Mills 1985; Prymak, McKee et al. 1988; Johnson, Powers et al. 1989) Surgery may be palliative to arrest active hemorrhage but does not provide long-term survival for the majority of the cases with visceral involvement.(Brown, Patnaik et al. 1985; Johnson, Powers et

al. 1989; Kerstetter, Krahwinkel et al. 1997; Wood, Moore et al. 1998)

Chemotherapy in the adjuvant setting has been evaluated in various clinical studies and has shown promise in prolonging life after surgical resection of the primary lesion.(MacEwen, Kurzman et al. 1994; Vail, MacEwen et al. 1995; Kim, Liptak et al. 2007; Lana, U'Ren et al. 2007; U'Ren, Biller et al. 2007) Most of those studies include dogs with different clinical stages. It appears that dogs with gross metastatic disease at the time of diagnosis carry significantly worse prognosis, with reported median survival durations between 68 and 136 days.(Sorenmo, Duda et al. 2000; Sorenmo, Baez et al. 2004; Kim, Liptak et al. 2007) The most effective chemotherapy protocols for the treatment of canine HSA are anthracycline - based. Doxorubicin and its analog epirubicin have been used as single agents in standard, or dose - intense fashion, in combination with cyclophosphamide, or with cyclophosphamide and vincristine.(Ogilvie, Reynolds et al. 1989; Hammer, Couto et al. 1991; Sorenmo, Jeglum et al. 1993; Ogilvie, Powers et al. 1996; Sorenmo, Duda et al. 2000; Sorenmo, Baez et al. 2004; Sorenmo, Samluk et al. 2007) There have been no studies evaluating specific treatment of dogs with grossly metastatic HSA, a common presentation in the majority of HSA - bearing dogs.(Brown, Patnaik et al.

1985; Ng and Mills 1985; Hammond and Pesillo-Crosby 2008) Non-resectable primary hemangiosarcoma appears to carry a negative prognosis for overall survival, and use of chemotherapy or palliative radiation therapy has been largely unexplored.(Aronsohn 1985)

Our study evaluated the effects of a combination chemotherapy protocol comprised of doxorubicin, dacarbazine, and vincristine (DAV) for the treatment of advanced stage canine HSA. Dogs were considered to have advanced stage disease when non-resectable primary tumor and/or gross metastatic disease was present at the time of diagnosis. Dacarbazine (DTIC) is an alkylating agent used in the treatment of relapsed round cell tumors, high-grade sarcomas, and malignant melanomas.(Gray, Raulston et al. 1984; Van Vechten, Helfand et al. 1990; Ahaus, Couto et al. 2000; Dervisis, Dominguez et al. 2007) Results of in vitro and in vivo studies suggest that DTIC acts synergistically with anthracyclines and has moderate effect in the treatment of high-grade sarcomas in humans.(Choi, Ng et al. 1985; Etcubanas, Horowitz et al. 1985; Elias and Antman 1986; Pearl, Inagami et al. 2002; Zucali, Bertuzzi et al. 2008) Vincristine has demonstrated modest response in a limited number of cancer patients.(Hahn 1990; Hammer, Couto et al. 1991) We, therefore, sought to evaluate the combination of

2008) Vincristine has demonstrated modest response in a limited number of cancer patients.(Hahn 1990; Hammer, Couto et al. 1991) We, therefore, sought to evaluate the combination of doxorubicin, DTIC, and vincristine in the setting of non-resectable or grossly metastatic HSA. We hypothesized that this combination will be effective in providing clinical responses with secondary goals of extended time to progression and overall survival in dogs with advanced stage HSA.

MATERIALS AND METHODS

Animals

Dogs examined at Michigan State University Animal Cancer Care Clinic between January 2004 and December 2006, with newly diagnosed hemangiosarcoma, were eligible for inclusion in the study. The study was designed and performed in compliance with the Michigan State University's Institutional Animal Care and Use Committee guidelines for research on animals, and owner informed consent was obtained before entering a dog in the study. Dogs diagnosed with hemangiosarcoma were included only if they had not received any type of chemotherapy treatment. In addition, dogs were included only if they had measurable disease and the diagnosis had been confirmed on the basis of results of histologic or cytologic examination. For the cytologic examination to be consistent with the diagnosis of hemangiosarcoma, all the following criteria had to be satisfied: cytologic diagnosis of sarcoma; cytologic diagnosis of pathologic hemorrhage; and confirmation of multi-cavitated lesion via ultrasonographic examination.(Nguyen and Husain 2000; Delacruz, Jorda et al. 2005; Gagner, Yim et al. 2005) Dogs were considered to have a high risk of developing doxorubicin-related

cardiotoxicosis if systolic fractional shortening determined echocardiographically was < 25%. These dogs were excluded from the study. Dogs with life-limiting non-neoplastic co-morbid conditions were excluded from the study.

Treatment Protocol

Doxorubicin and DTIC were administered on day 1, while vincristine was administered on days 8 and 15. The protocol was repeated every 21 days, to a maximum of 6 cycles. Doxorubicin was administered through an indwelling IV catheter over 20 minutes at a dose of 30 mg/m^2 diluted in 50-100 mls of 0.9% NaCl. DTIC was administered immediately after doxorubicin administration through the same catheter at a dose of 800 mg/m^2 over 8 hours, diluted in a volume of 0.9% NaCl equal to 17.6 mls/kg [8 ml/lb]. Pretreatment with dexamethasone sodium phosphate (4mg IV) and butorphanol (0.4 mg/kg [0.88 mg/lb] IM) was performed before each DTIC treatment. Metoclopramide (0.5 mg/kg [1.1 mg/lb]) was dispensed to the owners to be administered PO as needed at home. Vincristine was administered through a butterfly catheter IV as a bolus, at a dose of 0.5 mg/m^2.

Dose reductions were allowed only for DTIC in the case of grade III or IV toxicity, in 100 mg/m^2 increments. No dose escalations were allowed for any drug.

Evaluation of Response and Toxicity

In all dogs, a CBC and complete physical examination were performed on days 1, 7, 15, and 22 of the treatment. An echocardiographic evaluation of cardiac function was performed before the first doxorubicin/ DTIC administration. An electrocardiogram was performed before each subsequent treatment with doxorubicin/ DTIC to monitor for changes associated with doxorubicin-induced cardiotoxicity.(Danesi, Del Tacca et al. 1989; Noda, Watanabe et al. 1998) In addition, thoracic radiography and abdominal ultrasonography were performed every 2 treatment cycles (6 weeks) to define response to treatment. Any evidence of progressive primary disease or new ultrasonographic lesions was investigated by means of cytologic examination of fine-needle aspirates. Lesions appearing cavitated under ultrasonographic examination were considered to be positive for HSA, even when cytology was negative for neoplasia. Dogs were considered lost to follow-up when not returned for scheduled recheck examinations and the veterinarian or owner could not be

contacted despite repeated attempts; dogs lost to follow-up were censored.

Tumor response was determined each time dogs were examined. A complete response was defined as disappearance of all measurable disease for at least 21 days. A partial response was defined as > 50% but < 100% reduction in the measurable disease for at least 21 days. Stable disease was defined as < 50% reduction in measurable disease for at least 21 days with no appearance of new lesions during that period. Progressive disease was defined as > 25% increase in measurable disease or appearance of new lesions. Transient decreases in measurable disease that persisted for < 21 days and were followed by increased tumor size, were defined as progressive disease.

Toxicoses were identified on the basis of history and results of physical examinations and CBCs. Criteria established by the Veterinary Cooperative Oncology Group were used to grade toxicoses.(2004)

Statistical analysis

The study was structured with a 2-stage design that would allow early study closure if the overall clinical benefit rate was unacceptably low.(Simon 1989; Simon 1989) In the first stage, 10 patients were entered. If 2 or more

clinical responses were seen, then an additional 12 patients were entered into the study for a planned total of 22 cases. If 7 or more clinical responses were observed in total, then the conclusion was that the chemotherapeutic protocol was effective; less than 7 responses in the total patient sample population indicated insufficient anticancer activity of the DAV protocol to support continued evaluation. This study is designed for a Type I error of 0.05, and a Type II error of 0.1, with sufficient power to distinguish a clinically promising response rate of 0.5 from an unpromising response rate of 0.2.

Time to progression (TTP) was defined as the time from initiation of the chemotherapy treatment until disease progression. Disease free interval (DFI) was defined as the time from the initiation of the chemotherapy treatment until disease relapse for those dogs that achieved complete remission. Overall survival (OS) time was defined as the time from initiation of the chemotherapy protocol until patient death. The Kaplan-Meier survival analysis method was used to estimate response and survival time curves following treatment. The Log-Rank test was used to compare the effect of potential risk factors (gender, age, body weight, stage, median dose of DTIC, DTIC dose reductions, splenic primary site, lung metastasis on presentation,

peripheral blood hematocrit before treatment, and peripheral blood platelet number before treatment) to DFI, TTP, OS. The Cox proportional-hazards regression method was used to determine whether potential risk factors (gender, age, body weight, stage, median dose of DTIC, DTIC dose reductions, splenic primary site, lung metastasis on presentation, peripheral blood hematocrit before treatment, and peripheral blood platelet number before treatment) were associated with time to disease progression or overall survival time following chemotherapy. The potential risk factors were entered in the regression model if their $p<0.05$ and removed if $p>0.1$.

All reported *P* values are 2-sided. Values of $P < 0.05$ were considered significant. Statistical analyses were performed with standard software.

RESULTS

Patients

Twenty-four dogs were enrolled in the study, with 14 male and 10 female dogs. Breeds represented in the study were: German shepherds (7), Golden retrievers (6), Labrador retrievers (2), Beagle (1), American Pitt Bull terrier (1), English setter (1), and mixed breed (6). The mean age of all dogs was 10.4 years (± SD, 1.8) and mean body weight was 32.4 kg (± SD, 8.4). Twenty dogs had evidence of metastatic HSA upon presentation and four dogs had large, non-resectable primary hemangiosarcoma. Of the twenty dogs with stage III HSA, 15 had surgical resection of the primary tumor and attempted resection of the metastasis, with 3 achieving resection of all gross primary and metastatic disease. Five dogs with metastatic disease were characterized as poor surgical candidates and were treated only with chemotherapy. Four dogs with stage II disease did not undergo surgical resection of their primary tumor, due to tumor size and location (Table 2).

A median of 3.5 cycles (range, 1 - 6) of DAV chemotherapy were administered, with 79 cycles administered in total. The median cumulative dose of doxorubicin was 90

mg/m^2 (range, 30 - 180). The median DTIC administered dose was 785.5 mg/m^2 (range, 500 - 800) and the median cumulative dose was 2,600 mg/m^2 (range, 500 - 4,600). The median cumulative dose of vincristine was 3.5 mg/m^2 (range, 0.5 - 6).

Efficacy

Nineteen dogs were evaluable for response, while 3 dogs had no evidence of disease after surgery and were treated in an adjuvant setting, and 2 dogs were lost to follow up. Five of 19 dogs had a complete response, and 4/19 dogs had a partial response. Nine of 19 dogs had stable disease while 1/19 had progressive disease during the course of DAV treatment.

The median disease free interval (DFI) for dogs that achieved CR was 205 days (range, 82 - 400). The median time to progression (TTP) for dogs that either had a clinical response or had stable disease was 101 days (range, 21 - 400) (Figure 1). The median overall survival (OS) for all dogs in the study was 125 days (range, 18 - 411) (Figure 2).

All but 2 animals were euthanized due to progressive HSA. One dog was euthanized due to acute renal failure

while in CR, and the other dog was found dead by the owners 1 day after a complete restaging (complete blood count, serum chemistry, urinalysis, thoracic radiographs, abdominal ultrasound) demonstrated CR. The owners of both these dogs declined necropsy. Two of the 4 dogs with advanced stage II disease failed locally and were euthanized due to local disease progression 214 and 282 days after starting DAV, while the other 2 developed metastatic disease and were euthanized 71 and 125 days after starting DAV chemotherapy.

Toxicity

The most common treatment - related toxicoses observed were hematologic and gastrointestinal. Overall, 221 events of hematologic toxicity were noted. Of these toxicities, 23 were grade III and 18 were grade IV. Grade III toxicities included anemia (n=9), neutropenia (n=12), and thrombocytopenia (n=2). Grade IV toxicities included anemia (n=4), neutropenia (n=10), and thrombocytopenia (n=4). A total of 96 events of gastrointestinal toxicoses were observed. Of these events, 8 were grade III, and 4 were grade IV toxicities. Grade III toxicities included anorexia (n=1), emesis (n=3), and diarrhea (n=4). Grade IV toxicities included emesis (n=2), and diarrhea (n=2).

The mean hematocrit of the treated dogs at the beginning of their therapy with the DAV protocol was 36.1% (± 5.16), and at the end of the treatment protocol 33.6% (±, 7.8) [reference range 40 - 55%]. The mean platelet number of the treated dogs at the beginning of their treatment with the DAV protocol was 382 x 10^3 / µl (± 237 x 10^3), and at the end of the treatment protocol was 451 x 10^3 (±, 179 x 10^3) [reference range 155 - 393 x 10^3]. These differences were not statistically significant.

Four out of 24 dogs were hospitalized due to side effects of the chemotherapy for a median of 4 days (range 2 - 9). The hospitalized dogs had concurrently hematologic and gastrointestinal high grade toxicosis. All dogs manifested the severe toxicoses after the doxorubicin/DTIC treatment. No dogs died or were euthanized due to treatment related toxicoses, but three of these dogs discontinued chemotherapy as a result of the adverse effects. Additionally, one dog discontinued treatment due to toxicity, as assessed by its owner, despite this dog did not need to be hospitalized.

Risk factor analysis

Univariate analysis of risk factors indicated that dogs with non-splenic primary HSA had a favorable median DFI of 307 days versus dogs with splenic primary HSA that achieved a median DFI of 104 days ($p = 0.0136$). The median dose of DTIC administered was positively associated with longer TTP ($p = 0.0041$). Dogs that had to be treated with a reduced dose of DTIC due to toxicity, had longer median OS (211 days) when compared with dogs that did not require dose reductions (median 100 days) ($p = 0.0159$). The median dose of DTIC was positively associated with longer duration of OS ($p = 0.004$). Dogs that stopped chemotherapy treatment early due to toxicity had a median OS of 79.5 days as compared to dogs in which the protocol was well tolerated and achieved median OS of 151 days ($p = 0.0252$). Dogs that had metastatic disease to the lung parenchyma upon diagnosis had a median OS of 67 days, as compared to dogs presenting with metastatic disease outside of the lung parenchyma that achieved a median OS of 151 days ($p = 0.0365$).

Multivariate analysis identified age (RR 2.3, $p = 0.049$) to be negatively associated with TTP (Figure 3) while DTIC dose reductions (RR 0.06, $p = 0.031$) were positively associated with TTP (Figure 4). DTIC dose

reductions was the sole factor positively associated with OS (RR 0.28, p = 0.015).

Additional treatments

Six dogs received ifosfamide rescue treatment upon tumor progression and 18 dogs did not receive any rescue chemotherapy treatment. No response was seen in the ifosfamide treated dogs. Two dogs with advanced stage II disease received palliative radiation therapy (3 weekly 8 Gy fractions using 6 MV photons) but demonstrated no clinical improvement of local disease. One dog finished 6 cycles of the DAV protocol in CR and continued to be treated with a combination of dactinomycin and temozolomide once a month until tumor relapse, for a total of 12 treatments. Four dogs finished the DAV protocol in CR and were included in a phase I study of a metronomic chemotherapeutic protocol. One of these dogs was euthanized due to acute renal failure while in CR, and the other one was found dead by the owners 1 day after being completely staged as free of disease.

DISCUSSION

Dogs with HSA have a poor prognosis, with only a relatively small percentage of dogs achieving long-term survival.(Ogilvie, Reynolds et al. 1989; Hammer, Couto et al. 1991; Sorenmo, Jeglum et al. 1993; Ogilvie, Powers et al. 1996; Sorenmo, Duda et al. 2000; Sorenmo, Baez et al. 2004; Kim, Liptak et al. 2007; Lana, U'Ren et al. 2007) Dogs presenting with advanced clinical stage disease are considered to have grave prognosis, and many times are not offered any treatment options other than hospice or euthanasia. In our study, we demonstrate the activity of a combination chemotherapy protocol that is based on a doxorubicin / DTIC administration on the same day, combined with vincristine administration. Our results indicate that even dogs with widespread metastatic or non-resectable HSA can benefit from aggressive doxorubicin - based chemotherapy, achieving a CR rate of 26.3% and a PR rate of 21.1%, for a total response rate of 47.4%. Despite the overall poor prognosis in this disease setting, the complete responders achieved a median DFI of 205 days. A minority of dogs survived for a longer period, with 10% of the dogs being alive at 10 months from the beginning of the treatment, and only one surviving beyond one year.

The patients with the longest survival were those that achieved CR either through chemotherapy alone, or started treatment in an adjuvant setting after complete surgical removal of all gross primary and metastatic disease. The difference in survival between dogs that were treated in an adjuvant setting and those treated in the gross disease setting was not statistically significant, presumably due to the small number of dogs in the adjuvant treatment group (N=3). Formal power analysis in our patient sample indicates that, in patients with stage III HSA, to detect a difference in survival between animals treated with aggressive surgical resection of all primary and metastatic disease versus animals treated with conservative surgical resection of only the bleeding tumors and followed by chemotherapy, with alpha of 0.05 and power of 80%, twenty dogs are required for each group. Thus a larger study would be required to assess the validity of our observation.

One interesting and unexpected finding in our study was the positive association of DTIC dose reductions with increased TTP and OS. While in theory, lower doses of chemotherapy are associated with decreased cancer cell killing, we believe that our study results reflect the need to treat advanced stage HSA with the maximum tolerated dose (MTD) of DTIC. DTIC is a pro-drug that requires enzymatic

activation through members of the cytochrome-P450 system (CYP1A1, CYP1A2, and CYP2E1) to exert its cytotoxic effect. There is evidence indicating that genetic polymorphisms in these CYP genes can have profound effect in the pharmacokinetics of CYP-depended drugs.(Mise, Hashizume et al. 2004; Mise, Yadera et al. 2004; Tenmizu, Endo et al. 2004; Kamimura 2006; Tenmizu, Noguchi et al. 2006; Trepanier 2006) Therefore, it is possible that some dogs tolerate higher doses of DTIC than others, due to slower activation of the drug and subsequent smaller area under the drug concentration - time curve. Due to our study design, we did not escalate the DTIC dose, so we could not assess the individual patients MTD if they tolerated the standard dose of 800 mg/m^2. On the other hand, animals that demonstrated significant toxicity were subsequently treated with lower doses of DTIC, achieving this way a treatment closer to their individual MTD, which reduced the apparent risk of disease progression. Pharmacogenomic and pharmacokinetic studies of DTIC and its metabolites (MTIC, AIC) in the canine cancer patient would be invaluable to derive future dosing schema.

The toxicity of the DAV protocol appears to be significant. Five dogs were withdrawn from the study by their owners due to hematological and gastrointestinal

toxicity. All toxic events were observed after the doxorubicin/DTIC administration. None of these 5 dogs received any type of additional chemotherapy after the toxicity events and their median OS was significantly less than the rest of the patients in the study. Two of these dogs were lost to follow up and no data regarding their tumor response were available. Two dogs achieved PR with only 1 treatment of doxorubicin/DTIC, and one had SD. One of the dogs achieving PR had non-resectable primary right atrial HSA (OS: 241 days), and the other one had retroperitoneal HSA with widespread lung metastases (OS: 73 days).

Our study was designed as a single arm phase II, with the primary endpoint being the response rate, and secondary endpoints of frequency and severity of treatment - related toxicities and identific TTP, DFI, and. The study design required a minimum of 22 dogs in total, but we decided to include additional 2 dogs that presented to our clinic to increase the power of the study. The OS data should be evaluated with care, since six of the dogs that had PD or that relapsed during treatment with the DAV protocol received rescue treatment with ifosfamide. Four dogs that finished the DAV protocol in CR, were assigned to a metronomic protocol as part of an independent phase I

study. Additionally, the OS is defined from the start of the DAV protocol and not from the time of surgery or the time of diagnosis. Thus, while no dog responded favorably to rescue treatments and the effect of our metronomic treatment is difficult to assess at this time, the OS data should be interpreted under the aforementioned conditions.

Our study had a number of limitations. While we tried to have a homogeneous sample of animals with HSA carrying the worst prognosis, it is well understood by the authors that including dogs with advanced stage II disease, might increase the biological variability in the study. Additionally, a small number (N=3) of dogs in the study had only cytologic diagnosis of HSA. All these dogs had tumors that were difficult to obtain tissue samples for histopathologic diagnosis, we thus we relied on a cytologic diagnosis with strong clinical and imaging criteria that pointed towards HSA. It is possible that some of these tumors were other types of high-grade metastatic sarcomas, but their response to treatment, TTP, and OS, were not significantly different to those of the histopathologically - confirmed HSA dogs thus we decided to include these cases in our analysis.

Further studies are required to evaluate the best way to treat canine HSA. While it is evident that doxorubicin

at standard dosing is probably the basis of the most effective treatment options available today, phase III clinical trials are needed to compare the efficacy and toxicity of different doxorubicin - based protocols in a large population of dogs with distinct clinical stages of the disease.

Figure 2: Time to progression of patients with advanced stage HSA achieving CR, PR or SD with doxorubicin/DTIC/vincristine (N=18).

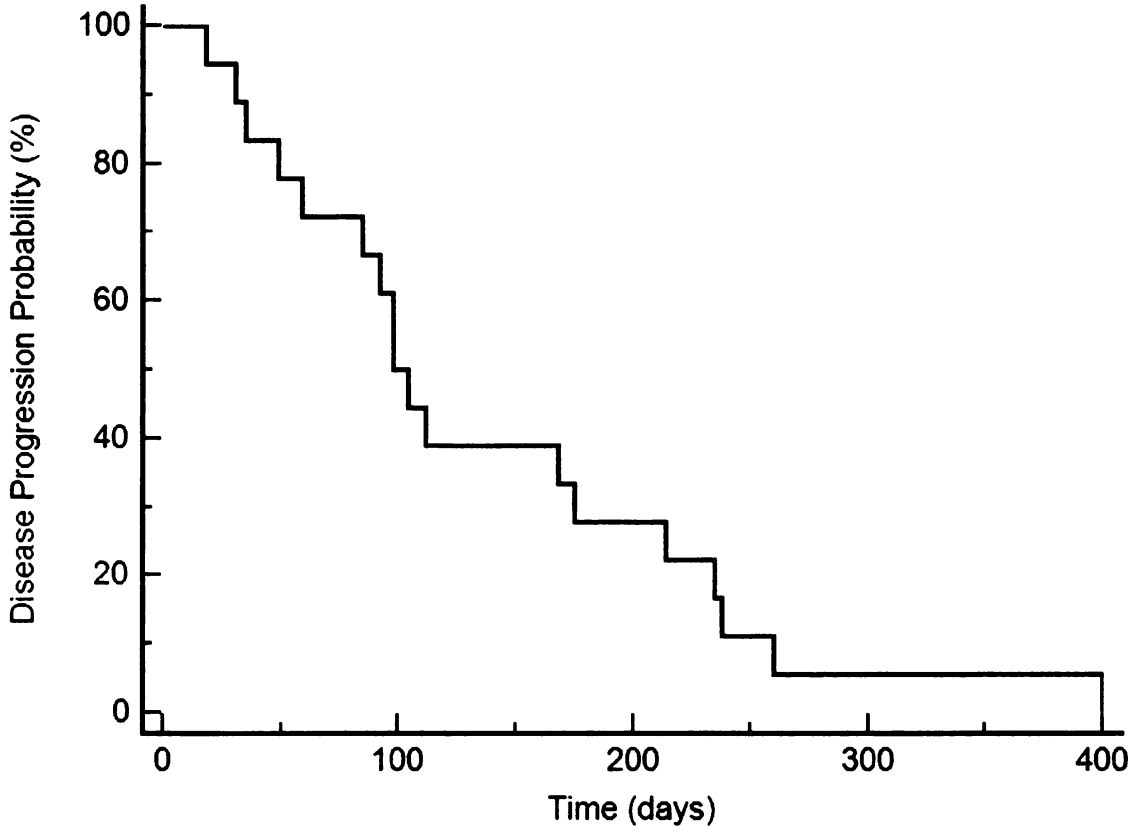


Figure 3: Overall survival of patients with advanced HSA treated with doxorubicin/DTIC/vincristine (N=24).

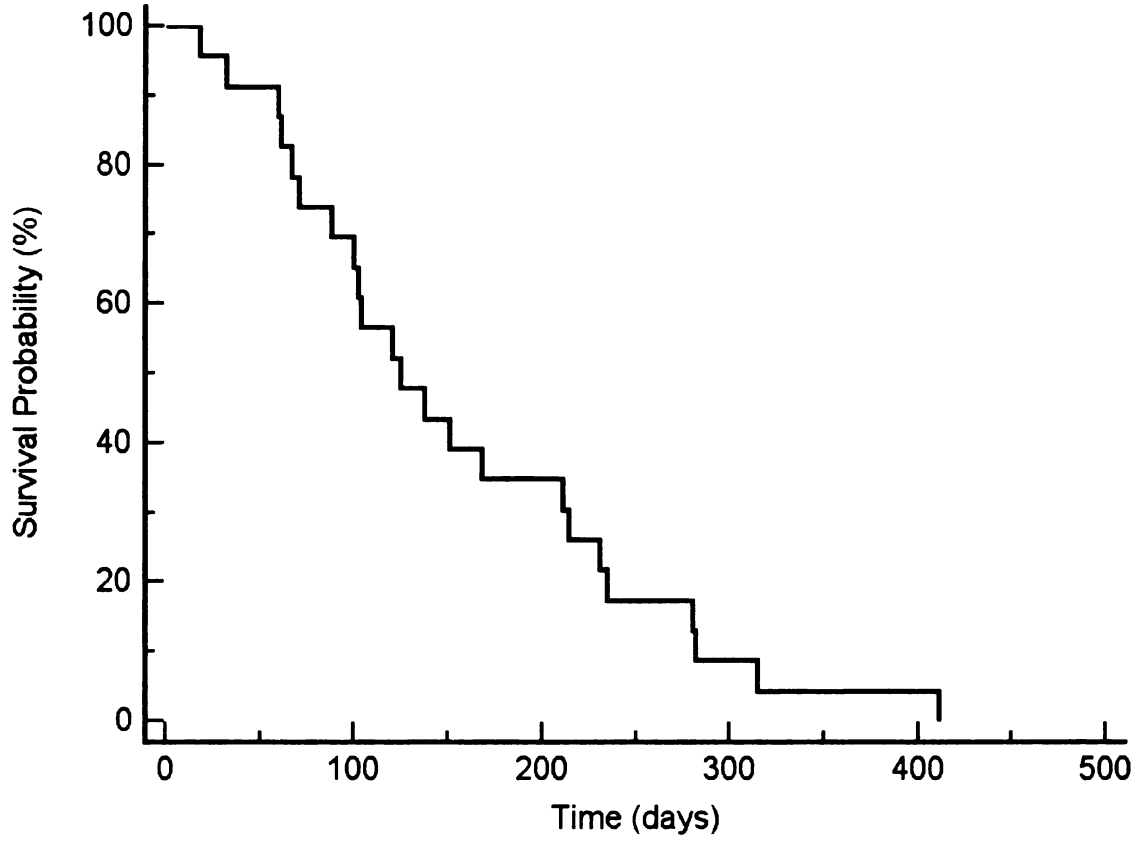


Figure 4: Age as a risk factor (RR=2.3, p=0.049) for disease progression in dogs with advanced HSA treated with doxorubicin/DTIC/vincristine. Dotted line represents the 75-100% age quartile (11.5-14.2 years), dashed-dotted line represents the 50-75% age quartile (10.3-11.5 years), solid line represents the 25-50% age quartile (9.7-10.3 years), and the dashed line represents the 0-25% age quartile (5.5-9.7 years).

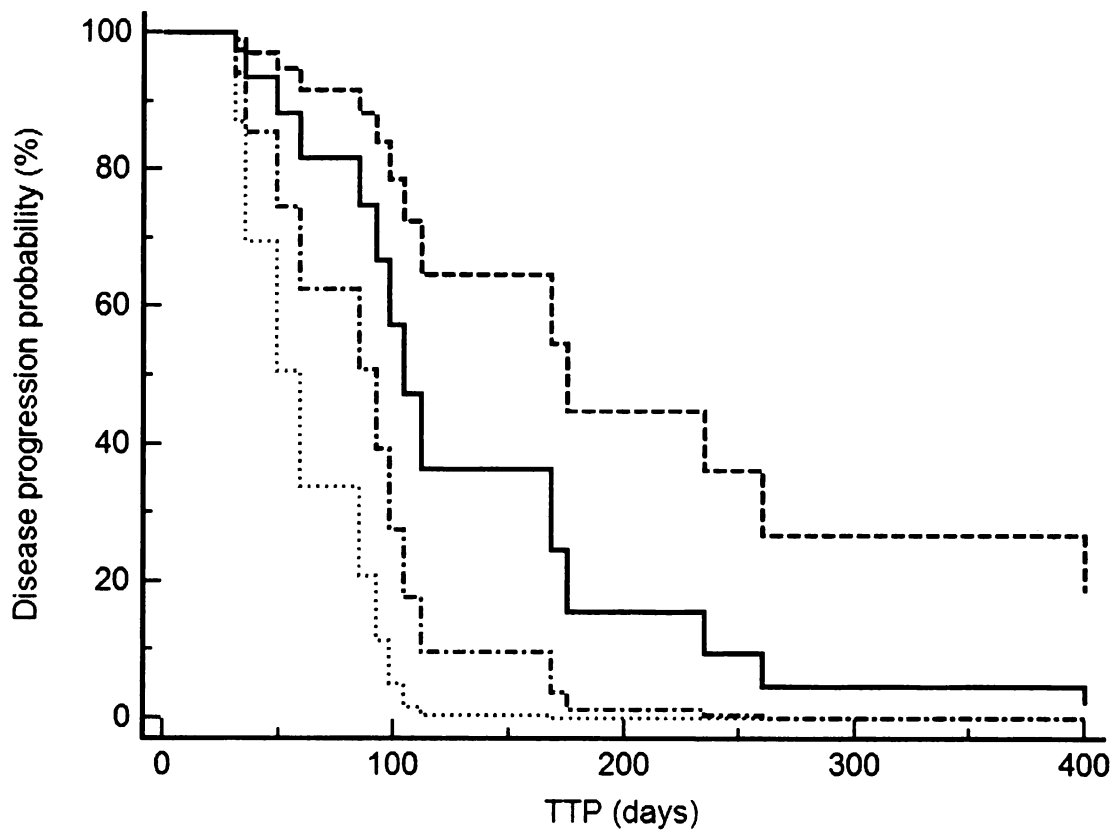


Figure 5: DTIC dose reductions due to toxicity reduced the risk of disease progression (RR=0.06, p=0.031). Solid line represents patients having DTIC dose reductions due to toxicity; dashed line represents patients without DTIC dose reductions.

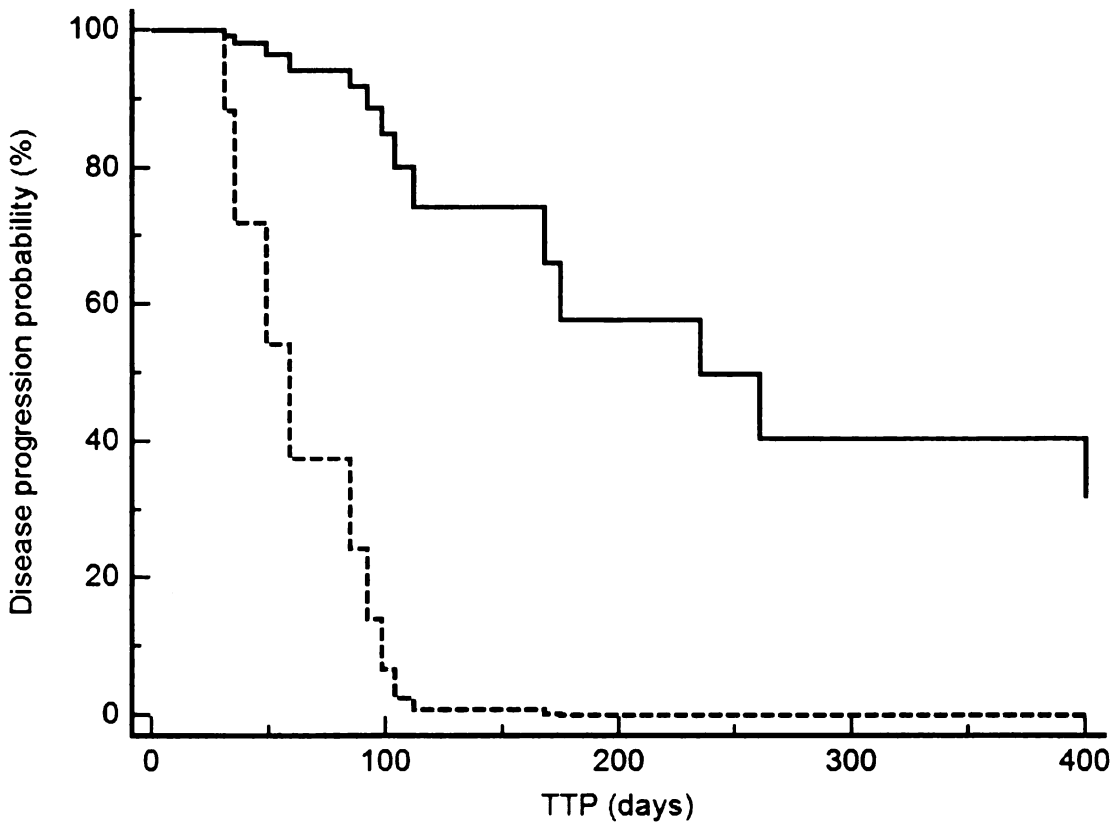


Table 1: Localization of primary tumors and metastatic sites upon presentation and upon tumor progression during treatment.

Organ	Primary site /(%)	Metastatic sites on presentation / (%)	Metastases sites on treatment failure / (%)
Spleen	12 / (50%)	1 / (4.25%)	1 / (3.3%)
Liver	1 / (4.25%)	9 / (37.5%)	8 / (26.6%)
Lungs	1 / (4.25%)	7 / (29.5%)	4 / (13.4%)
Mesentery	0 / (0%)	1 / (4.25%)	0 / (0%)
Omentum	0 / (0%)	2 / (8%)	2 / (6.7%)
Heart	3 / (12.5%)	0 / (0%)	3 / (10%)
Central nervous system	0 / (0%)	0 / (0%)	2 / (6.7%)
Bone	2 / (8%)	1 / (4.25%)	1 / (3.3%)
Gastrointestinal tract	0 / (0%)	0 / (0%)	1 / (3.3%)
Subcutaneous	3 / (12.5%)	1 / (4.25%)	4 / (13.4%)
Eye	0 / (0%)	0 / (0%)	1 / (3.3%)
Vagina	1 / (4.25%)	0 / (0%)	0 / (0%)
Retroperitoneum	0 / (0%)	2 / (8%)	0 / (0%)
Kidney	1 / (4.25%)	0 / (0%)	1 / (3.3%)
Lymph node	0 / (0%)	0 / (0%)	2 / (6.7%)
TOTAL	24 / (100%)	24 / (100%)	30 / (100%)

CHAPTER 3

Efficacy of temozolomide or dacarbazine in combination with an anthracyclines for rescue chemotherapy in dogs with lymphoma

INTRODUCTION

Treatment of relapsed or refractory lymphoma in dogs often involves administration of an anthracycline, such as doxorubicin or dactinomycin, in combination with an alkylating agent. Dacarbazine is an alkylating agent that has been used in the treatment of relapsed round cell tumors, high-grade sarcomas, and malignant melanomas,(Van Vechten, Helfand et al. 1990) and results of in vitro and in vivo studies(Leikin, Bernstein et al. 1975; Remmelink, Salmon et al. 1997) suggest that dacarbazine acts synergistically with anthracyclines in humans. Dacarbazine has been administered in combination with doxorubicin or dactinomycin for treatment of relapsed lymphoma in dogs,(Van Vechten, Helfand et al. 1990; Mellanby, Herrtage et al. 2003) and this combination appears to have moderate antineoplastic activity. However, its use has been limited by the requirement that dacarbazine

be administered as a slow IV infusion over 8 hours or as a bolus by slow IV injection once daily for 5 consecutive days every 3 weeks.(Gray, Raulston et al. 1984; Ahaus, Couto et al. 2000) In addition, dacarbazine has been associated with clinically important gastrointestinal tract toxicoses, both acute and delayed, and hematologic toxicoses, including neutropenia and thrombocytopenia, and accidental extravasation may cause local irritation and pain.(Van Vechten, Helfand et al. 1990; Mellanby, Herrtage et al. 2003)

Temozolomide is a recently developed imidazotetrazine derivative that is essentially an oral formulation of dacarbazine.(Stevens, Hickman et al. 1984) Whereas dacarbazine must be activated by the cytochrome P-450 system in the liver, temozolomide is metabolized to its active form by a nonenzymatic pathway in the blood.(Stevens, Hickman et al. 1987; Tsang, Quarterman et al. 1991) Thus, we speculate that the response to treatment would be better with temozolomide, compared with dacarbazine, because of elimination of the variable introduced by cytochrome P-450 activation necessary for dacarbazine activity. Further, we hypothesize that the toxicity of dacarbazine is influenced by pharmacogenomic factors introduced by variations in cytochrome P-450 activity among individuals and that toxicity would thus be minimized by use of temozolomide.(Tsang,

Quarterman et al. 1991; Reid, Kuffel et al. 1999) Finally, because temozolomide is hydrolyzed to a common intermediate of the dacarbazine activation pathway, we suspect that temozolomide might have pharmacokinetic and clinical benefits in relation to dacarbazine.

The purpose of the study reported here, therefore, was to compare results of treatment with temozolomide or dacarbazine, in combination with an anthracycline, in dogs with relapsed or refractory lymphoma.

MATERIALS AND METHODS

Dogs

Dogs examined at the Michigan State University Animal Cancer Care Clinic between January 2004 and December 2006 because of relapsed or refractory lymphoma were eligible for inclusion in the study. Dogs with relapsed lymphoma were included only if they had received induction treatment with a multidrug chemotherapy protocol that included doxorubicin; dogs with refractory lymphoma were included only if they had not responded to a standard induction chemotherapy protocol. In addition, dogs were included only if they had measurable disease and the diagnosis had been confirmed on the basis of results of histologic or cytologic examination. Dogs with non-neoplastic co-morbid conditions were excluded from the study.

Group assignment

Two treatment groups (temozolomide and an anthracycline vs. dacarbazine and an anthracycline) were included in the study. At the time of the study, the cost of treating a dog with a combination of temozolomide and an anthracycline was approximately twice the cost of treating a dog with a combination of dacarbazine and an anthracycline. Therefore,

dogs were not randomly assigned to treatment groups. Rather, treatment group assignments were made on the basis of owner cost concerns. Owners of all dogs included in the study provided informed consent, and the study was performed in compliance with guidelines for research in animals established by the Michigan State University All-University Committee on Animal Use and Care.

Within each treatment group, dogs were treated with doxorubicin or dactinomycin on the basis of perceived risk of developing doxorubicin-related cardiotoxicosis. Patients evaluated as being at high risk for cardiotoxicosis were switched to dactinomycin instead of doxorubicin. Dogs were considered to have a high risk of developing doxorubicin-related cardiotoxicosis if systolic fractional shortening determined echocardiographically was < 25% or if total prior cumulative doxorubicin dose was ≥ 180 mg/m^2.

Treatment protocol

Doxorubicin was diluted in 50 to 100 mL of 0.9% NaCl and administered through an indwelling IV catheter over 20 minutes at a dose of 30 mg/ m^2. Dactinomycin was also diluted in 50 to 100 mL of 0.9% NaCl and administered through an indwelling IV catheter over 20 minutes, but at a dose of 0.6

mg/m^2. Dacarbazine was administered immediately after anthracyclines administration through the same catheter at a dose of 800 mg/m^2; the calculated dose was diluted in 0.9% NaCl (17.6 mL/kg [8 mL/lb]) and administered over 8 hours. Dogs were treated with dexamethasone sodium phosphate (4 mg, IV) and butorphanol (0.4 mg/kg [0.18 mg/lb], IM) before each dacarbazine treatment, and metoclopramide (0.5 mg/kg [0.23 mg/lb], PO) was dispensed to be administered as needed at home. Temozolomide was dispensed to participating owners to be administered at home. Owners were instructed to wear latex gloves when handling the drug, to not open the capsules, and to not allow their animal to chew the capsules.

The initial intended dosage of temozolomide was 60 mg/m^2, PO, every 24 hours for 5 consecutive days, starting on the same day as anthracycline administration. For each successive group of 3 dogs, the dose was increased by a factor of 10 mg/m^2, up to an intended target dose of 100 mg/m^2, as long as none of the 3 dogs in the group developed toxicoses that required hospitalization. Because temozolomide was supplied as 5-, 20-, 100-, and 250-mg capsules, the calculated dose of temozolomide was rounded to the nearest 5 mg; reformulation was not done. Owners were instructed to

administer temozolomide on an empty stomach and to feed their dog 30 minutes later. Metoclopramide (0.5 mg/kg, PO) was administered 1 hour before temozolomide to prevent vomiting and as needed throughout the treatment period.

The chemotherapy treatment was repeated every 21 days in dogs that had a clinical response of at least 21 days' duration.

Evaluation of response and toxicoses

In all dogs, a CBC and complete physical examination were performed on the day of anthracycline administration and 7 and 21 days later at the Animal Cancer Care Clinic or by the referring veterinarian. In addition, thoracic radiography and abdominal ultrasonography were performed after every 2 to 3 treatment cycles in dogs that continued to respond. Any evidence of lymphadenopathy or ultrasonographic lesions was investigated by means of cytologic examination of fine-needle aspirates. Dogs were considered lost to follow-up when not returned for scheduled recheck examinations and the veterinarian or owner could not be contacted despite repeated attempts; dogs lost to follow-up were censored.

Tumor response was determined each time dogs were examined. A complete response was defined as disappearance of all measurable disease for 21 days. A partial response was

defined as > 50% but < 100% reduction in measurable disease for 21 days. Stable disease was defined as < 50% reduction in measurable disease for 21 days with no appearance of new lesions during that period. Progressive disease was defined as > 25% increase in measurable disease or appearance of new lesions. Transient decreases in measurable disease that persisted for < 21 days were defined as progressive disease.

Toxicoses were identified on the basis of history and results of physical examinations and CBCs. Criteria established by the Veterinary Cooperative Oncology Group were used to grade toxicoses.(2004)

Statistical analysis

Duration of first remission was defined as the time from initial administration of the original induction chemotherapy protocol until relapse. Duration of response to rescue chemotherapy was defined as the time from completion of the initial cycle of rescue chemotherapy until progression of disease. Survival time following rescue chemotherapy was defined as the time from completion of the initial cycle of rescue chemotherapy until death. Overall survival time was defined as the time from initial administration of the original induction chemotherapy protocol until death. The Kaplan-Meier survival analysis method was used to estimate

response and survival time curves following treatment. The log-rank test was used to compare Kaplan-Meier curves between treatment groups (temozolomide treatment group vs dacarbazine dactinomycin vs. doxorubicin followed by dactinomycin subgroup), and between responding and nonresponding dogs. Continuous variables (age and body weight) were tested by use of the D'Agostino-Pearson test to determine whether they were normally distributed. Continuous variables that were found to be normally distributed were compared between treatment groups by means of a 2-sample *t* test. The Pearson χ^2 test was used to determine whether the distribution of categoric variables (sex, stage, substage, immunophenotype, and presence of hypercalcemia) differed between treatment groups. The Cox proportional-hazards regression method was used to determine whether potential risk factors (i.e., sex, age, body weight, stage, substage, immunophenotype, and presence of hypercalcemia) were associated with duration of response to rescue chemotherapy or survival time following rescue chemotherapy. The potential association between dosage of temozolomide and duration of response to rescue chemotherapy was examined by use of the log-rank test for trends. The chi-square test with Yates' correction for continuity was used to determine whether treatment-related toxicoses were associated with treatment group.

All reported *P* values are 2-sided. Values of $P < 0.05$ were considered significant. Statistical analyses were performed with standard software.

RESULTS

Patients

Sixty-three dogs were enrolled in the study. Twenty-one dogs were treated with a combination of temozolomide and an anthracycline, and 42 were treated with a combination of dacarbazine and an anthracycline. A total of 55 cycles of the temozolomide-anthracycline protocol were administered, of which 41 included doxorubicin and 14 included dactinomycin. A total of 119 cycles of the dacarbazine-anthracycline protocol were administered, of which 48 included doxorubicin and 71 included dactinomycin.

Mean age of dogs in the temozolomide treatment group (mean ± SD, 10.1 ± 3.2 years) was not significantly (P = 0.47) different from mean age of dogs in the dacarbazine treatment group (8.5 ± 2.8 years). Similarly, mean body weight of dogs in the temozolomide treatment group (22.4 ± 12.3 kg [49.3 ± 27.1 lb]) was not significantly (P = 0.51) different from mean body weight of dogs in the dacarbazine treatment group (34.2 ± 10.9 kg [75.2 ± 24 lb]). Treatment groups did not differ with regard to sex, breed, initial stage, initial substage, immunophenotype, or presence of hypercalcemia at the time of initial examination (Table 1).

Previous treatment

Prior to enrollment in the present study, all dogs had been treated with a multidrug chemotherapy protocol (cyclophosphamide, doxorubicin, vincristine, prednisone, and l-asparaginase). Dogs in the dacarbazine treatment group had received a median of 5 chemotherapy drugs (range, 3 to 8). Dogs in the temozolomide treatment group had also received a median of 5 chemotherapy drugs (range, 3 to 10). All but 1 dog had received a combination of l-asparaginase, vincristine, cyclophosphamide, doxorubicin, and prednisone for induction chemotherapy. The remaining dog did not receive vincristine because of severe cholestasis and hypoalbuminemia.

Median duration of first remission prior to enrollment in the present study was 151 days (range, 26 to 320 days) for dogs in the temozolomide treatment group, with 14 of the 21 (67%) dogs relapsing during the initial induction protocol. Median duration of first remission prior to enrollment in the present study was 145 days (range, 16 to 321 days) for dogs in the dacarbazine treatment group, with 29 of the 42 (69%) dogs relapsing during the initial induction protocol. The temozolomide-anthracycline combination was the first rescue chemotherapy protocol for 12 of the 21 dogs in the temozolomide treatment group, the second rescue chemotherapy

protocol for 3 dogs, the third rescue chemotherapy protocol 5 dogs, and the fifth rescue chemotherapy protocol for 1 dog. The dacarbazine-anthracycline combination was the first rescue chemotherapy protocol for 34 of the 42 dogs in the dacarbazine treatment group, the second rescue chemotherapy protocol for 5 dogs, and the third rescue chemotherapy protocol for 3 dogs.

Temozolomide treatment group

Median number of treatment cycles for dogs in the temozolomide treatment group was 2 (range, 1 to 8). Median dosage of temozolomide was 92.6 mg/ m^2 (range, 60.6 to 103.9 mg/ m^2), and median cumulative dose was 953 mg/ m^2 (range, 305.1 to 3,692 mg/ m^2).

Eighteen dogs were evaluated for response, while 3 dogs were lost to follow-up. Median duration of response to rescue chemotherapy for the 18 dogs in the temozolomide treatment group was 40 days (range, 0 to 217 days). Thirteen dogs (72%) had a complete (n = 9) or partial (4) response, and median duration of response for these dogs was 65 days (range, 23 to 217 days). The remaining 5 dogs (28%) had progressive disease, with duration of response recorded as 0 days for all 5 dogs. Sixteen dogs received temozolomide in combination

with doxorubicin, 3 dogs received temozolomide in combination with dactinomycin, and 2 dogs initially received temozolomide in combination with doxorubicin but were switched to dactinomycin after they reached a cumulative doxorubicin dose of 180 mg/ m^2. No significant (P = 0.76) difference was found between these 3 subgroups (doxorubicin vs. dactinomycin vs. doxorubicin followed by dactinomycin) with regard to duration of response.

Median survival time following rescue chemotherapy for the 18 dogs in the temozolomide treatment group was 72 days (range, 30 to 352 days), with 3 dogs still alive at the time of the study. Median overall survival time (i.e., time from initial administration of the original induction chemotherapy protocol until death) was 230 days (range, 87 to 646 days). Median overall survival time for the 13 dogs that had a complete or partial response to rescue chemotherapy was 251 days (range, 89 to 646 days), whereas median survival time for the 5 dogs that did not have a complete or partial response to rescue chemotherapy was 166 days (range, 87 to 464 days). Median overall survival time was not significantly (P = 0.27) different between these 2 subgroups.

Dacarbazine treatment group

Median number of treatment cycles for dogs in the dacarbazine treatment group was 2 (range, 1 to 9). Median dose of dacarbazine was 800 mg/ m^2 (range, 450 to 800 mg/ m^2), and median cumulative dose was 1,400 mg/ m^2 (range, 584 to 6,300 mg/ m^2). Thirty-five dogs were evaluated for response, while 7 dogs were lost to follow-up. Median duration of response to rescue chemotherapy for the 35 dogs in the dacarbazine treatment group was 50 days (range, 0 to 587 days). Twenty-five dogs (71%) had a complete (n = 22) or partial (3) response, and median duration of response for these dogs was 85 days (range, 34 to 587 days). Seventeen dogs received dacarbazine in combination with doxorubicin, 22 dogs received dacarbazine in combination with dactinomycin, and 3 dogs initially received dacarbazine in combination with doxorubicin but were switched to dactinomycin after they reached a cumulative doxorubicin dose of 180 mg/ m^2. No significant (P = 0.27) difference was found between these 3 subgroups (doxorubicin vs. dactinomycin vs. doxorubicin followed by dactinomycin) with regard to duration of the response.

Median survival time following rescue chemotherapy for the 35 dogs in the dacarbazine treatment group was 104 days

(range, 10 to 587 days), with 4 dogs still alive at the time of the study. Median overall survival time was 238 days (range, 57 to 688 days). Median overall survival time for the 25 dogs that had a complete or partial response to rescue chemotherapy was 337 days (range, 127 to 688 days), whereas median survival time for the 10 dogs that did not have a complete or partial response to rescue chemotherapy was 105 days (range, 57 to 188 days). Median overall survival time was significantly ($P < 0.001$) different between these 2 subgroups.

Toxicoses

For dogs in both treatment groups, the most common toxicoses were hematologic and gastrointestinal. Overall, for dogs in the temozolomide treatment group, there were 15 episodes of grade I hematologic toxicosis, 6 episodes of grade II hematologic toxicosis, 2 episodes of grade III hematologic toxicosis (thrombocytopenia), and 1 episode of grade IV hematologic toxicosis (neutropenia). Only 1 dog required hospitalization as a result of hematologic toxicosis (neutropenic sepsis and sinusitis), and in 3 instances, the dosage of temozolomide was reduced because of hematologic toxicoses. Gastrointestinal tract toxicoses (nausea,

vomiting, and diarrhea) that occurred were self-limiting, and no dogs required hospitalization.

There were 14 episodes of grade I, 7 episodes of grade II, and 1 episode of grade IV (nausea) gastrointestinal tract toxicosis. In 2 dogs, treatment with temozolomide was discontinued because of the high cost. Two dogs became azotemic, 1 after the second treatment and the other after the fifth treatment. The former dog also had preexisting heart disease and was receiving mexiletene and enalapril; this dog was euthanized because of progressive renal failure while in remission. The other dog that developed azotemia received another 3 cycles of treatment, after which the lymphoma relapsed; the azotemia did not progress during this period. Another dog had episodes of urinary incontinence 2 to 3 days after the first day of treatment with temozolomide. Results of microbial (bacterial, mycoplasmal, and ureoplasmal) culture were negative, and the dog regained urinary continence after every treatment cycle.

Overall, for dogs in the dacarbazine treatment group, there were 79 episodes of grade I hematologic toxicosis, 28 episodes of grade II hematologic toxicosis, 18 episodes of grade III hematologic toxicosis, and 17 episodes of grade IV hematologic toxicosis (neutropenia and thrombocytopenia). Eight dogs had to be hospitalized because of neutropenic

sepsis and dehydration, and 1 dog had to be hospitalized because of neutropenic sepsis and petechiation secondary to thrombocytopenia. The latter dog was euthanized after 2 days of hospitalization because of toxicoses. Five of the dogs hospitalized because of neutropenia had concurrent grade III (n = 4) or grade IV (1) gastrointestinal tract toxicoses (nausea, vomiting, and diarrhea). In 16 instances, the dosage of dacarbazine was reduced because of hematologic toxicoses. In 1 dog, treatment with dacarbazine was discontinued, and in 2 dogs, treatment was changed to temozolomide because of toxicoses. There were 20 episodes of grade I, 21 episodes of grade II, 7 episodes of grade III, and 2 episodes of grade IV gastrointestinal tract toxicosis.

Dogs in the temozolomide treatment group had a total of 24 episodes of hematologic toxicosis (0.44 episodes/treatment), whereas dogs in the dacarbazine treatment group had a total of 142 episodes of hematologic toxicosis (1.19 episodes/treatment). The incidence of high-grade (ie, grade III or IV) hematologic toxicoses was significantly ($P < 0.001$) higher among dogs in the dacarbazine treatment group than among dogs in the temozolomide treatment group. However, the incidence of gastrointestinal tract toxicoses did not differ significantly between groups.

Risk factor analysis

There were no significant differences between treatment groups in regard to proportion of dogs with a complete or partial response, duration of response to rescue chemotherapy (Figure 1), survival time following rescue chemotherapy, or overall survival time. Sex, age, body weight, stage, substage, and hypercalcemia status were not found to be significantly associated with duration of response to rescue chemotherapy or overall survival time.

For dogs in the temozolomide treatment group, multivariate analysis indicated that stage was significantly (P = 0.02) associated with duration of response to rescue chemotherapy (Figure 2), that age (P = 0.02) and stage (P = 0.03) were significantly associated with survival time following rescue chemotherapy, and that immunophenotype was significantly (P = 0.02) associated with overall survival time. When dogs were grouped on the basis of anthracyclines administration (doxorubicin vs. dactinomycin vs. doxorubicin followed by dactinomycin), there were no significant differences among groups with regard to proportion of dogs with a complete or partial response, duration of response to rescue chemotherapy, survival time following rescue chemotherapy, overall survival time, incidence of hematologic toxicoses, or incidence of gastrointestinal tract toxicoses.

For dogs in the dacarbazine treatment group, multivariate analysis indicated that body weight was significantly ($P = 0.01$) associated with survival time following rescue chemotherapy and that body weight ($P = 0.005$), hypercalcemia status ($P = 0.03$), and immunophenotype ($P = 0.04$) were significantly associated with overall survival time.

When dogs were grouped on the basis of anthracycline administration (doxorubicin vs dactinomycin vs. doxorubicin followed by dactinomycin), there were no significant differences among groups with regard to proportion of dogs with a complete or partial response, duration of response to rescue chemotherapy, survival time following rescue chemotherapy, overall survival time, incidence of hematologic toxicoses, or incidence of gastrointestinal tract toxicoses.

DISCUSSION

Results of the present study suggested that administration of temozolomide at a dose of up to 100 mg/m^2 in combination with an anthracycline every 3 weeks appeared to be well tolerated in dogs with relapsed or refractory lymphoma. In particular, the incidence of hematologic toxicoses was significantly lower in dogs given the temozolomide-anthracycline combination, compared with dogs given a combination of dacarbazine and an anthracycline, although there was no difference in incidence of gastrointestinal tract toxicoses between treatment groups. Both combinations had promise in the treatment of dogs with relapsed or refractory lymphoma, although administration of temozolomide was more convenient and less toxic than administration of dacarbazine.

The present study was primarily designed to evaluate the toxicity of temozolomide when administered in combination with an anthracycline, and tumor response was a secondary end point. Dacarbazine was used as the comparator because the active forms of temozolomide and dacarbazine are essentially identical with regard to mechanism of action.(Tsang, Quarterman et al. 1991) Bioavailability of temozolomide following oral administration in dogs is 100%, and there is

no clinically important metabolism or accumulation of the drug in the body.(Kim, Lin et al. 1997) The main route of temozolomide excretion in dogs is through the kidneys, with minimal excretion taking place through the feces or respiratory tract.

Dogs appear to be more sensitive to temozolomide than humans, in that the maximum nonlethal dose for dogs (200 mg/m^2) is similar to the therapeutic dose for humans. In a chemoprotection study, dogs were given temozolomide at doses as high as 800 mg/m^2, but the bone marrow was protected in these dogs by transplantation with stem cells transfected with specific drug resistance genes.(Neff, Beard et al. 2005) In dogs given lethal doses, signs of potential CNS toxicosis were observed, including high body temperature, nausea, and vomiting. Toxic effects in dogs given nonlethal doses primarily involved the bone marrow, lymphoid tissues, testes, and gastrointestinal tract, as would be expected with an alkylating agent. No cardiovascular effects or renal abnormalities were detected in dogs treated with temozolomide in toxicology studies, and the carcinogenic potential of temozolomide appears to be similar to that of dacarbazine.

In human studies, administration of temozolomide with food resulted in a 33% decrease in maximum serum

concentration and a 9% decrease in the area under the concentration-versus-time curve. Although the clinical importance of these changes is unclear, it has been suggested that temozolomide be administered on an empty stomach.(Newlands, Blackledge et al. 1992; Dhodapkar, Rubin et al. 1997) In the present study, therefore, we advised owners to give temozolomide on an empty stomach and to offer food 30 minutes later to allow for maximum absorption of the drug while limiting gastric irritation.

In the present study, dogs were assigned to treatment groups solely on the basis of the owner's ability to afford the more expensive treatment. We believe that this reduced the bias associated with allowing clinicians to assign cases to treatment groups. However, owners willing to invest more financially might have been more inclined to delay euthanasia in the event of treatment failure, adding some degree of bias to survival outcomes in our study.

In contrast, dogs were assigned to receive doxorubicin or dactinomycin on the basis of objective criteria (i.e., total cumulative dose of doxorubicin received and echocardiographic evidence of impaired systolic function). Dogs that reached a cumulative dose of doxorubicin of 180 mg/m^2 were considered to be at high risk of developing cardiotoxicosis and were automatically switched to

dactinomycin to minimize any potential contribution of doxorubicin toxicity in assessing the toxicity of temozolomide and dacarbazine.(Loar and Susaneck 1986; Hammer, Couto et al. 1991)

The maximum nonlethal dose of temozolomide in healthy male Beagles has been reported to be 200 mg/m^2. We chose to use a dose of 60 mg/m^2 as a starting point in the present study on the basis of available toxicology data for dogs, the minimum risk of toxicoses when administered at this dose in humans, and the convenience of administration given the currently available capsule sizes. In addition, we chose a conservative initial dose because this was, to our knowledge, the first evaluation of temozolomide in tumor-bearing dogs. Dose escalation in cohorts of 3 dogs resulted in a final median dose of 92.6 mg/m^2.

One dog in the present study had episodes of urinary incontinence a few days after each cycle of temozolomide-dactinomycin administration was begun. These episodes lasted throughout the 5-day period of temozolomide administration during each treatment cycle. During each episode, results of a urinalysis and microbial culture of urine samples were negative. The dog was treated with antimicrobials each time, and signs regressed within 1 to 2 days after the last

temozolomide dose was given in each cycle. In a study of 158 human patients treated for anaplastic astrocytoma with temozolomide as the sole agent, 13% of the patients experienced urinary incontinence.(Yung, Prados et al. 1999) Thus, it is possible that neurologic toxicosis was the reason for the urinary incontinence in this dog.

Two dogs in the temozolomide-anthracycline treatment group in the present study developed azotemia during treatment. One dog had preexisting heart disease characterized by arrhythmias and was being treated with mexiletene, taurine, and enalapril. The dog had a complete response but became azotemic after the second treatment cycle. The dog was reevaluated echocardiographically and the dosage of enalapril was reduced. However, the azotemia progressed, and the owners elected to euthanize the dog. The other dog developed mild azotemia after the fifth treatment cycle. The azotemia did not progress in this dog, and the dog received an additional 3 cycles of treatment.

Results of the present study suggested that a combination of temozolomide and an anthracycline may be useful in the treatment of dogs with relapsed or refractory lymphoma. The greater ease of administration and lower toxicity were attractive features when temozolomide was compared with dacarbazine. Although duration of response was

modest in the present study, 43 of 63 (68%) dogs accrued had refractory disease that relapsed during the course of frontline doxorubicin-based induction therapy. These dogs would typically be expected to have shorter durations of response to rescue chemotherapy than the 3- to 5-month rescue remission durations expected for dogs that relapsed after completion of the induction chemotherapy protocol.(Calvert and Leifer 1981; Hohenhaus and Matus 1990; Moore, Ogilvie et al. 1994; Ahaus, Couto et al. 2000; Rassnick, Mauldin et al. 2002; Alvarez, Kisseberth et al. 2006) In addition, because of the dose escalation aspect of the study, some dogs received relatively low doses of temozolomide.

Although we did not find a significant relationship between temozolomide dose and duration of response to rescue chemotherapy, the statistical power for detecting such a relationship was low. Also, we used strict criteria for terminating the dose escalation of temozolomide because chemotherapy in these dogs was largely intended to be palliative. Additional dose escalation studies are needed to establish the maximum tolerated dose of temozolomide when administered in combination with anthracyclines in dogs with cancer. Treatment of dogs with lymphoma before refractory disease is established might also be expected to improve

outcomes, and the addition of temozolomide to induction chemotherapy protocols merits further investigation.

Table 2: Characteristics of dogs included in the study

CHARACTERISTICS	# OF PATIENTS	
	DTIC arm (N = 42)	**TMZ arm** (N = 21)
Age (years)		
Median	8.0	10.1
Mean	8.5	10.9
Range	3.5-15.2	3.0-16.5
Gender		
Male	21	9
Female	21	12
Weight (kg)		
Median	35.2	22.4
Mean	34.2	21.5
Range	10.2-70.4	5.8-48.9
Breeds		
Mix	11	6
Golden	9	3
Labrador	6	0
Boxer	5	1
Other purebred	11	11
Initial WHO stage		
III	14	5
IV	18	9
V	10	7
Initial WHO substage		
a	27	11
b	15	10
Hypercalcemia upon initial presentation		
Yes	5	4
No	37	17
Immunophenoptype		
B	23	8
T	7	7
Non-B/non-T	2	0
Unknown	10	6

Figure 6: Kaplan - Meier analysis of duration of the response to rescue treatment. 1 → DTIC arm, 2 → TMZ arm. Animals not evaluated for response are censored from the analysis.

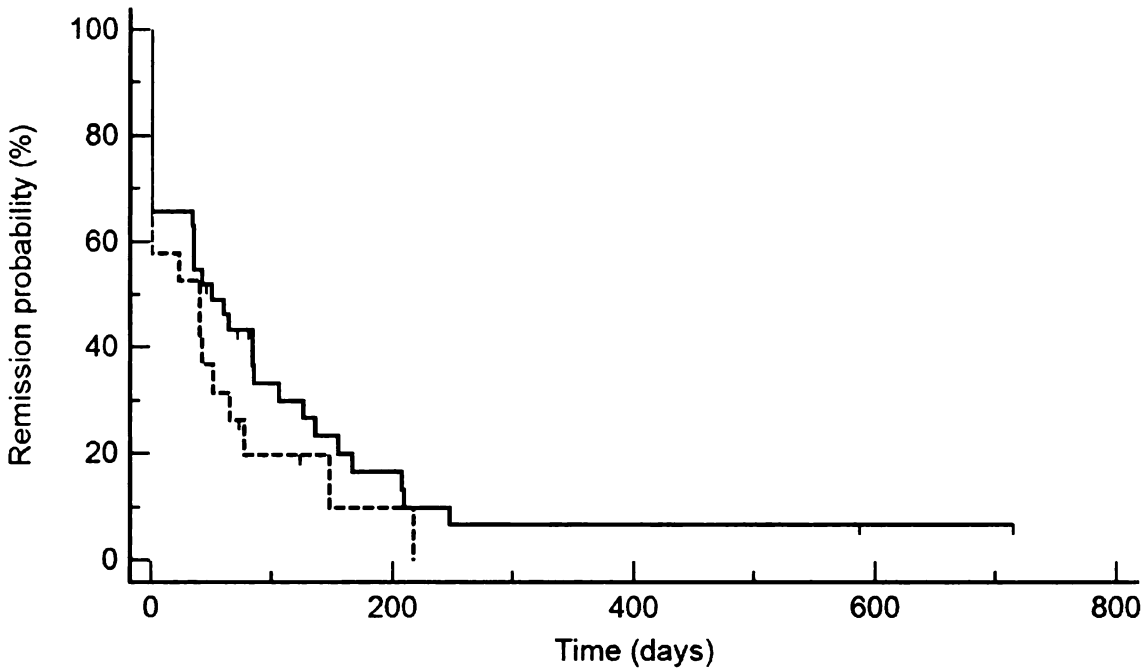
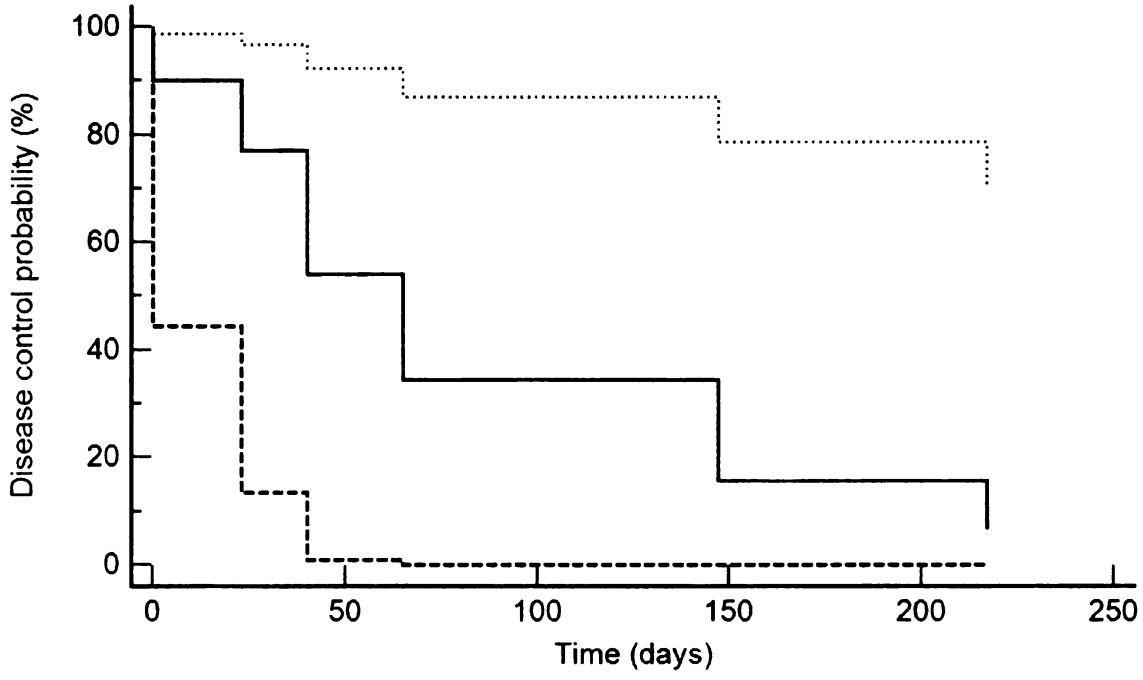


Figure 7: Multivariate analysis of the impact of disease stage on duration of rescue for all dogs in the TMZ arm ($p = 0.02$). 3 → WHO stage III, 4 → WHO stage IV, 5 → WHO stage V.



CHAPTER 4

Variation in cytochrome P450 genes responsible for dacarbazine activation in the dog

INTRODUCTION

Cancer is the leading cause of death in older companion animals. In an autopsy series of 2000 dogs, 23% of all dogs, regardless of age, and 45% of dogs over 10 years old, were determined to have died of cancer. (Vail and MacEwen 2000) Estimates of age-adjusted overall cancer incidence rates per 100,000 individuals / years at risk range from 243 to 381. These rates are comparable to those reported by the National Cancer Institute SEER program for humans. (Hansen and Khanna 2004) Chemotherapy is administered based on the assumption that each individual will metabolize drugs with the same efficiency. Most dogs tolerate multidrug chemotherapeutic protocols well, but some patients experience serious or lethal adverse effects despite the standardized drug dosing and scheduling. (Ahaus, Couto et al. 2000; Mellanby, Herrtage et al. 2003)

Many drugs used in veterinary oncology are substrates for phase I or phase II metabolizing enzymes. Cytochrome P450

(CYP) superfamily enzymes are critical in the phase I metabolism of drugs, catalyzing primarily oxidative reactions. Many chemotherapeutic drugs, such as dacarbazine, cyclophosphamide, ifosfamide, paclitaxel, docetaxel, and vincristine, are depended upon CYP-catalyzed activation in order to exert their tumoricidal effect. (McFadyen, Melvin et al. 2004)

Dacarbazine (DTIC) is a chemotherapeutic agent used in human and veterinary oncology in the treatment of refractory lymphoma, melanoma, and sarcoma, despite the lack of full understanding of its pharmacokinetic profile in the canine cancer patient. (Loo, Tanner et al. 1968; Dervisis, Dominguez et al. 2007) DTIC is a prodrug that requires enzymatic conversion to form the linear triazine 3-methyl-(triazen-1-yl) imidazole-4-carboxamide (MTIC). (Tsang, Quarterman et al. 1991) The activation process in humans is believed to be primarily dependent on *CYP1A2*, *CYP2E1*, and *CYP1A1*. (Reid, Kuffel et al. 1999)

Temozolomide (3,4-dihydro-3-methyl-4-oxoimidazo [5,1-d]-alpha-tetrazine-8-carboxamide) is a recently developed imidazotetrazine derivative that is essentially an oral formulation of DTIC. (McFadyen, Melvin et al. 2004) The active metabolite of temozolomide is MTIC, the same as DTIC. However, temozolomide is metabolized to MTIC in the

peripheral blood by a non-enzymatic pathway. (Loo, Tanner et al. 1968; Loo, Housholder et al. 1976) It has been hypothesized that the absence of any enzymatic mechanism of metabolism may reduce inter-animal dose variability. (Dervisis, Dominguez et al. 2007)

Observation of toxic adverse reactions in dogs receiving anticancer chemotherapy is relatively common in clinical veterinary oncology practice.(Moore and Kitchell 2003) In certain disease treatment settings, strong suspicions have been raised with regard to potential inter-animal variability in the administered drug metabolism. Data from two recent studies from our group strongly support this.

In a prospective clinical trial completed at our clinic we compared dogs treated with an DTIC based protocol versus dogs treated with a temozolomide based protocol. Results demonstrated similar efficacy of the two chemotherapeutic protocols, but the incidence of hematologic high-grade toxicity events more frequent in the DTIC arm versus the temozolomide arm. (Dervisis, Dominguez et al. 2007) The toxicity profile of the DTIC arm of our study is in agreement with previously published data. (Ahaus, Couto et al. 2000) The same frequency of serious adverse effects in dogs treated with a DTIC based protocol for a type of metastatic high sarcoma was observed in a different prospective clinical

study conducted in our clinic (paper under editorial review). This observation led us to the hypothesize that differences in the metabolic activation of DTIC between individuals can account for significant variability in the safety of certain drugs, such as DTIC. To test this hypothesis can, the tools to detect genetic variability in the genes responsible for DTIC activation must be developed.

Few data are available for specific CYP expression and activity in the dog. While over 60 CYP genes are believed to exist, only nine have been characterized in part in the dog.(Graves, Elhag et al. 1990; Uchida, Komori et al. 1990; Ciaccio, Graves et al. 1991; Sakamoto, Kirita et al. 1995; Fraser, Feyereisen et al. 1997; Blaisdell, Goldstein et al. 1998; Lankford, Bai et al. 2000) Even though there is a significant lag in the canine CYP characterization, it appears that large interspecies differences might exist for many CYP enzymes. (Walton, Dorne et al. 2001) Additionally, CYP variants corresponding to poor and extensive metabolizers have been demonstrated in the dog, with the most notable example being the presence of *CYP1A2* mutants in research beagle colonies. (Mise, Hashizume et al. 2004; Tenmizu, Endo et al. 2004; Tenmizu, Noguchi et al. 2006) The present study was designed to evaluate potential genetic variation in the

critical genes responsible for the DTIC activation, *CYP1A1*, *CYP1A2*, and *CYP2E1*, in the client-owned canine population.

MATERIALS AND METHODS

Study samples

Genomic DNA samples from our laboratory's DNA bank were used for the study. Dog breeds used in our study were German shepherd (N=8), Labrador retriever (N=8), Greyhound (N=8), Siberian Husky (N=274), Alaskan Malamute (N=28), Beagle (N=36), Scottish terrier (N=9), English pointer (N=6), Doberman pinscher (N=9), Collie (N=8), Cocker spaniel (N=8), and mixed breed (N=2).

Amplification reactions

Intronic primers, flanking each exon were designed using the canine genomic sequence information obtained though the UCSC Genome Browser[1] and the Primer3 software (Rozen and Skaletsky 2000; Kent, Sugnet et al. 2002). Primer sets were analyzed for primer loops, primer dimers and primer-primer compatibility using the OligoAnalyzer 1.0.2 software. Polymerase chain reaction conditions and primers used are summarized on Table 1. Amplification reactions were systematically evaluated and optimized. The PCR mixtures (25 µl) contained 1 µl template genomic DNA (20 nM), 2.5 µl 10X

[1] http://genome.ucsc.edu/

PCR buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl), 1 µl $MgCl_2$ (50 mM), 2 µl dNTPs (5 mM), 1 µl of forward and reverse primer (5 µM), 0.1 µl Taq polymerase (5 U/µl), and 16.4 µl deionized distilled water. The PCR profile was 3 min at 94°C, followed by 40 cycles of 45 sec at 94°C, 30 sec at 60°C, 90 sec at 72°C, and final extension of 10 min at 72°C. The PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide, and imaged by a Gel Doc 2000 Biorad system.

Sequencing analysis

All the PCR products were directly sequenced using the appropriate forward and reverse primers. An ABI 3730xl high through-put capillary DNA sequencer was used for the sequencing reactions. The obtained sequence chromatograms were evaluated for quality using the reported quality score and direct examination of the chromatogram traces. Sequence alignment and identification of potential polymorphic sites was performed using the LaserGene software suite. The presence of a distinct signal from the sequencing reaction of all the PCR products at the putative site indicated that the animal was homozygous for the mutation, while if N was

reported the animal was considered to be heterozygous for the polymorphism. Putative polymorphic sites were recorded.

Amplified fragment length polymorphism analysis

Amplified fragment length polymorphism analysis was performed to confirm all putative SNPs identified by direct sequencing. Online software was used to design restriction digestion assays that would distinguish between two alleles (Bikandi, San Millan et al. 2004). The results of each assay design were tested using NEBcutter v2.0 (Vincze, Posfai et al. 2003). In cases where no restriction enzyme was commercially available, mutagenic primers were used to create a restriction site that would distinguish between the two putative alleles (Bikandi, San Millan et al. 2004). All restriction enzymes and buffers were purchased from New England Biolabs. Restriction enzymes used for each SNP are summarized in Table 2.

The restriction digestion reactions were performed according to the manufacturer's recommendations. The digested PCR products were electrophoresed in 3.5% agarose gel, or in 20% polyacrylamide gel, depending on the expected fragment size. The gels were stained with ethidium bromide, and imaging was performed using a Gel Doc 2000 Biorad system.

Statistical Analysis

The risk of a SNP resulting in an amino acid change significant enough to alter the function of enzyme was calculated using the PolyPhen software (Ramensky, Bork et al. 2002). Chi-square analysis was performed to test if a significant difference in allele frequency exists between different breeds of dogs for each identified SNP.

RESULTS

CYP1A1

A total of 6 SNPs were identified: three SNPs in exon 1, and three SNPs in exon 6 (Table 3a). One SNP is a synonymous change (c.1362T>A), while five are nonsynonymous, leading to missense change in the protein sequence (c.53C>G → p.A18G; c.146G>T → p.W49L; c.345C>G → p.F115L; c.C1305C>A → p.F435L; and c.1561T>C → p.S521P).

The polymorphisms c.53C>G, c.345C>G and c.1305C>A were identified in heterozygous state only in Greyhounds, for a minor allele frequency of 7%, 14%, and 7% respectively in this breed. The polymorphic site at position 1561 appeared to be variable between breeds. The C-allele was the major allele in German shepherds and Siberian huskies, in contrast with Scottish terriers and Doberman pinscher s, where it was absent.

CYP1A2

A total of 6 SNPs were identified. Four of these SNPs have been already described (c.1117C>T; c.1173C>G; c.1299C>T; and c.1303G>A), while 2 novel SNPs were identified (c.1165A>G; and c.1451A>G) (Mise, Hashizume et al. 2004;

Tenmizu, Endo et al. 2004; Tenmizu, Noguchi et al. 2006). Of the novel SNPs, one is a synonymous change (1451A>G), and one is nonsynonymous leading to a missense change (c.1165A>G → p.N389D). The c.1117C>T, a known destructive change to the enzyme's function indentified in laboratory beagle dogs, was detected only in client beagles with a minor allele frequency of 16% (Table 3b).

A potential InDel was identified at the 6th exon in 7/13 Siberian huskies initially assayed. Sequencing of the cloned potential InDel - containing exon revealed a 10 base long duplication (1464_1473dupCCCCATCTAT). All 7 dogs harboring the duplication, appeared to be heterozygotes for it, hence we expanded our screening of the duplication to a set of a total of 274 Siberian husky genomic DNA. We identified 75 homozygotes for the wild type, and 199 dogs that appeared heterozygotes for the duplication. When we assayed 28 Alaskan malamutes for the duplication identified in the Siberian huskies, we failed to identify any dog positive for this duplication.

CYP2E1

A total of 2 SNPs in the coding region and 2 intronic SNPs were identified in the CYP2E1 gene. The 2 exonic SNPs

are nonsynonymous leading to missense change in the protein sequence (c.85C>T → p.R29C; and c.1453T>C → p.Y485H). The two intronic SNPs are located at the 5^{th} (IVS5+5G>A), and the 6^{th} intron, (IVS6+27C>T).

DISCUSSION

The results of our study support our hypothesis of significant genetic variation in the coding region of the CYP enzymes responsible for the activation of the anticancer drug DTIC. To the best of our knowledge, our study represents the first effort to characterize genetic variability in specific CYP enzymes, in client owned dogs of various breeds. Our results indicate significant interbreed and intrabreed variability, with a major allele in one breed being the minor allele in a different breed. Specific SNPs result in amino acid changes that might cause alteration in the activity or substrate specificity of the *CYP* genes studied. The impact of the identified SNPs could be significant, not only in the metabolism of dacarbazine, but also in the metabolism of other drugs, including the activation efficiency of pro-carcinogens to full carcinogens.

While our study did not focus on direct effect of identified SNPs to the cytochrome P450 function, we estimated the potential of each SNP causing significant functional alterations to each gene studied using the PolyPhen software. This prediction software has been evaluated extensively, and may provide valuable info on future functional studies on SNPs. When used for identification of deleterious

nonsynonymous SNPs from tolerant nonsynonymous SNPs in human *CYP* genes, prediction accuracy analysis indicated that 70% of the SNPs were predicted correctly as damaging (Wang, Li et al. 2009).

PolyPhen analysis indicates that in the *CYP1A1* gene, 3 SNPs have relatively high probability of functional disruption of the CYP1A1 activity (c.345C>G → p.F115L, PSIC score of 1.938; c.1305C>A → p.F435L, PSIC score of 2.871; and c.1561T>C → p.S521P, PSIC score of 1.604). No significant new SNPs were identified in the *CYP1A2* gene via PolyPhen analysis. Examining the *CYP2E1* gene indicates that the c.85C>T change may have high probability of affecting the enzyme's activity (PSIC score of 2.378).

The interbreed variability observed for some of SNPs might be important for future pharmacology studies in dogs, but also in epidemiologic studies of carcinogenesis. There are several reports in human literature implicating genetic polymorphisms with increased risk for certain types of cancer. *CYP1A1* and *CYP1A2* variants have been implicated in increasing the risk of chemical carcinogen induced cancers, such as lung, colon, and bladder cancer (Le Marchand, Hankin et al. 2001; Gago-Dominguez, Bell et al. 2003; Lee, Kang et al. 2008; Aldrich, Selvin et al. 2009). *CYP2E1* genetic

variants have been associated with increased risk for colorectal, acute lymphoblastic leukemia (Ulusoy, Adali et al. 2007; Morita, Le Marchand et al. 2009).

Of particular interest is the apparent duplication at the 6^{th} exon of *CYP1A2* (1464_1473dupCCCCATCTAT) in Siberian huskies. We were unable to identify homozygotes for the duplication, despite screening 274 dog DNA samples. This duplication appears to result in a frameshift and subsequent formation of a premature STOP codon, predicted to be located in the catalytic subunit of the CYP1A2 protein, potentially resulting in an inactive enzyme. While it can be hypothesized that abolishment of CYP1A2 activity would be a lethal trait for the bearing dogs, mice experiments indicate, that *CYP1A2* knockouts are phenotypically normal (Diliberto, Burgin et al. 1999; Smith, Davies et al. 2003). We were unable to identify any homozygote subjects for the duplication, with 75 dogs being homozygotes for the wild type, and 199 appear to be heterozygotes for the duplication. Our results violate the Hardy - Weinberg equilibrium (χ^2 = 58.87, $p<0.0001$). An alternative hypothesis for our findings is that the entire *CYP1A2* gene has been duplicated in Siberian huskies, and one copy of the gene acquired this 10bp duplication.

The results of our study could help in personalizing drug dosing of dacarbazine to each particular patient. Future pharmacologic studies may correlate specific SNPs with dogs that appear to be fast or slow metabolizers, opening the era of dosing chemotherapeutics, not based only on the body surface area of the dog, but also on its genotype. In veterinary medical oncology, the currently observed risk for serious adverse effects for dacarbazine treatment is relatively high. The addition of genotyping techniques before the 1^{st} treatment can potentially reduce this risk to lower, more acceptable levels.

Table 3: Conditions, primers and amplicon sizes for PCR

Genes	Exon	Forward primer	Reverse primer	Betaine 20%	T_A (C)	Product size (bps)
CYP1A1	1	TCCTCATCTCTCCCCATCTG	ATCTCTGACCTGCTGGCCTA	+	58	979
	2-5	TAGGTCTGGGTTTGGCATCT	GTCTGAGGCTTGGCAGAGG	+	58	850
	6	GGTTCAACCCACTTGATGCT	ACCCAGACAGGCCAGGTA	+	58	245
CYP1A2	1	GCAACTTGACACTTGGCTTG	CAGATGTTCCAGAGGCTTCC	-	62	999
	2	CCATATAGGGTACGGCAAGG	AACAACAGGCATGGATGGAT	-	62	233
	3	CCTTCCTCACCTTGCATCAT	CAAAGAACCTGGGGATCTCA	-	62	173
	4	CCGGTTTGGTCTTCTGTGTT	GAGGTCTGAGCCAGATCCAG	-	62	241
	5	GAGACACACCCCAGTGTTCC	AGGCCCCATTCCTGAATTAT	-	62	240
	6	ATCCCGACTCCTCTGTTCCT	GTGATCCTTCCCCTCCCTCT	-	62	365
CYP2E1	1	GACTGGTCACCCTCCTTCCT	TGAATTAAATGCACCCAAACC	-	58	339
	2	GCCCACATCCTCCCTAAGTT	GGTTACCACCCCCTGGTATT	-	62	290
	3	GTGGGACCGACACCGAGT	GGGAGCCACCACCTATGAC	+	62	316
	4	GGCCCCACAAGCCTTTTA	CCCGCTTAGAGGGCAAAG	+	60	400
	5	ACCCTGTTGCATCTGGAAAC	GGACCGTCTGGGCGTTAG	-	60	344
	6	GACGGTTCCCTCTCGTACAC	GTTAAAGCCCAGATGCTCCA	-	62	289
	7	CCGCTGGGAATGCTCTATAA	CAGCCACATCTACCAAAGGA	-	60	363
	8	GGAAGCCCGTGTTCTGTTAC	CGTGGCAGAGAGCAGGAC	+	63	250
	9	AGGGCTCTGTGTCTTGACC	TATGCGGAAGCAGGAAGA	+	61	300

Table 4: Summary of conditions for genotyping

Genes	SNPs	Enzyme	NEB buffer	Temperature (C)	Mutagenic Primers	
					Forward	Reverse
CYP1A1	c.53C>G	Sequencing	-	-	-	-
	c.146G>T	Sequencing	-	-	-	-
	c.345C>G	BsmAI	4	55	-	-
	c.1305C>A	HinfI	4	37	-	-
	c.1362T>A	Sequencing	-	-	-	-
	c.1561T>C	AluI	4	37	-	-
CYP1A2	c.1117C>T	MspI	4	37	CCGGTTTGGTC TTCTGTGTT	GGGACAAAGG AGGTGTGCC
	c.1173C>G	HpyCH4IV	1	37	-	-
	c.1299C>T	RsaI	4	37	-	-
	c.1303G>A	AciI		37	-	-
	c.1451A>G	Sequencing	-	-	-	-
CYP2E1	c.85C>T	AluI	4	37	-	-
	c.1453T>C	Sequencing	-	-	-	-
	IVS5+5G>A	BsaAI	4	37	-	-
	IVS6+27C>T	BsmFI	4	65	-	-

Table 5: Allele frequencies for CYP1A1 SNPs. Asterisks indicate predicted damaging amino acid change by PolyPhen analysis, while # indicates statistically significant differences between breeds. 1→chi-square = 70.169, p < 0.0001; chi-square = 13.682, p = 0.0334; 3→chi-square = 88.573, p < 0.0001.

Breeds	c.53C>G[#1] p.A18G		c.146G>T p.W49L		c.345C>G[#2] p.F115L*		c.1305C>A p.F435L*		c.1362T>A p.G454G		c.1561T>C[#3] p.S521P*	
	C (%)	G (%)	G (%)	T (%)	C (%)	G (%)	C (%)	A (%)	T (%)	A (%)	T (%)	C (%)
Labrador	100	0	75	25	100	0	100	0	100	0	10	90
Scottish terrier	100	0	-	-	100	0	100	0	100	0	0	100
Beagle	100	0	-	-	100	0	100	0	100	0	11	89
Siberian Husky	100	0	94	6	100	0	100	0	100	0	92	8
German shepherd	100	0	100	0	100	0	100	0	100	0	80	20
Collie	100	0	100	0	100	0	100	0	100	0	42	58
Doberman pinscher	100	0	100	0	100	0	100	0	100	0	0	100
English pointer	100	0	-	-	100	0	100	0	100	0	17	83
Greyhound	93	7	100	0	86	14	93	7	100	0	17	83
Cocker spaniel	75	25	100	0	100	0	100	0	94	6	10	90

Table 6: Allele frequencies for CYP1A2 SNPs. The # indicates statistically significant differences between breeds. 1→chi-square = 19.286, p = 0.0073; 2→chi-square = 233.824, p < 0.0001. Screening for c.1165A>G by amplified fragment length polymorphism analysis was unsuccessful. The minor allele frequency obtained via sequencing was 21%.

Breeds	c.1117C>T[#] 1 R373*fs*X		c.1165A>G p.N389D		c.1173C>G p.T391T		c.1299C>T p.G433G		c.1303G>A p.A435T		c.1451A>G[#] 2 p.R484K	
	C (%)	T (%)	A (%)	G (%)	C (%)	G (%)	C (%)	T (%)	G (%)	A (%)	A (%)	G (%)
Labrador	100	0	-	-	63	37	69	31	87	13	39	61
Scottish terrier	100	0	-	-	40	60	100	0	75	25	15	85
Beagle	84	16	-	-	75	25	71	29	64	36	50	50
Siberian Husky	100	0	-	-	72	28	94	6	100	0	97	3
German shepherd	100	0	-	-	75	25	50	50	100	0	94	6
Collie	100	0	-	-	75	25	100	0	87	13	36	64
Doberman pinscher	100	0	-	-	50	50	100	0	100	0	6	94
English pointer	100	0	-	-	75	25	50	50	100	0	40	60
Greyhound	100	0	-	-	-	-	-	-	-	-	92	8
Cocker spaniel	100	0	-	-	50	50	100	0	100	0	38	62

Table 7: Allele frequencies for CYP2E1 SNPs. Asterisk indicates predicted damaging amino acid change by PolyPhen analysis, while # indicates statistically significant differences between breeds. 1→chi-square = 98.338, $p < 0.0001$; 2→chi-square = 37.877, $p < 0.0001$.

Breeds	c.85C>T[#1] p.R29C*		c.1453T>C[#2] p.Y485H		c.825+5G>A		c.967+27C>T	
	C (%)	T (%)	T (%)	C (%)	G (%)	A (%)	C (%)	T (%)
Labrador	17	83	89	11	100	0	56	44
Scottish terrier	100	0	91	8	28	72	-	-
Beagle	13	87	96	4	86	14	-	-
Siberian Husky	6	94	65	35	100	0	78	22
German shepherd	100	0	95	5	100	0	-	-
Collie	100	0	50	50	100	0	-	-
Doberman pinscher	83	17	60	40	100	0	88	12
English pointer	-	-	100	0	100	0	-	-
Greyhound	42	58	100	0	91	9	63	37
Cocker spaniel	83	17	100	0	100	0	71	29

Figure 8: Location of CYP1A1 amplicons on the canine chromosome 30. 1 → contains exon 1; 2-5 → contains exons 2, 3, 4, and 5; 6 → contains exon 6.

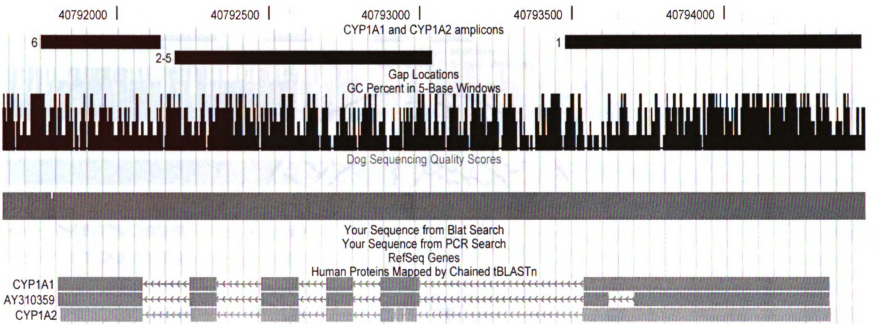
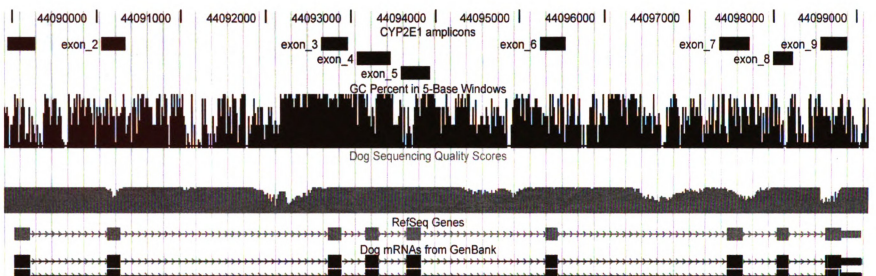


Figure 9: Location of CYP1A2 amplicons on the canine chromosome 30. 1 → contains exon 1; 2 → contains exon 2; 3 → contains exon 3; 4 → contains exon 4; 5 → contains exon 5; 6 → contains exon 6.

Figure 10: Location of CYP2E1 amplicons on the canine chromosome 28. 1 → contains exon 1; 2 → contains exon 2; 3 → contains exon 3; 4 → contains exon 4; 5 contains exon 5; 6 contains exon 6; 7 → contains exon 7; 8 → contains exon 8; 9 → contains exon 9.



CHAPTER 5

Importance of CYP genetics and future directions

Major advances in the treatment of cancer have resulted from the recent developments in medical and basic molecular biology research. However, significant heterogeneity in the efficacy and toxicity of chemotherapeutic agents is consistently observed across the human and pet animal population.(Evans and Relling 1999; Hahn, Ogilvie et al. 2008) Administration of the same dose of a specific anticancer drug to a population of patients results in a range of toxicity, from unaffected to lethal events.(Rothenberg, Meropol et al. 2001; Dervisis, Dominguez et al. 2007)

While many clinical variables have been associated with drug response (age, gender, diet, organ function, tumor biology), in both canine and human species, genetic differences in drug disposition and drug targets can have a great impact on treatment outcome.(Evans and Johnson 2001; Tenmizu, Noguchi et al. 2006) The metabolic enzymes and cellular targets for the majority of chemotherapy agents contain genetic polymorphisms but prospective identification of patients likely to benefit from, or be harmed by, chemotherapy is not currently possible for most treatments.

This is particularly important in the in the treatment of canine cancer, since the main goal for clinicians and pet owners is palliation of the disease symptoms and improvement of the patients quality of life.(Ahaus, Couto et al. 2000; Mellanby, Herrtage et al. 2003)

The importance of pharmacogenetics in personalized medicine is underlined by the FDA approval of the first pharmacogenetic test, the AmpliChip CYP450 Test, in 2005.(de Leon, Armstrong et al. 2006) The AmpliChip CYP450 Test assesses two polymorphic genes, the *CYP2D6* and the *CYP2C19*, using cDNA array technology.(Jain 2005) These two genes coding for enzymes responsible for the metabolism of various drugs, but most importantly for medications used in psychiatry. A recent paper indicated that current antipsychotics may be the ideal place for implementing pharmacogenetic techniques, since they are efficacious in only 30% of patients and tend to have a narrow therapeutic window.(Webster, Martin et al. 2004) Like modern psychiatric medicine, medical oncology is also faced with small efficacy of most chemotherapeutic drugs, which also have a very narrow therapeutic window.

Multiple examples of the clinical effect of CYP polymorphisms exist in human oncologic medicine. The activation of cyclophosphamide to 4-hydroxycyclophosphamide,

which is mediated mostly by CYP2B6 and CYP3A4, could be affected by polymorphisms in the corresponding genes. This was confirmed in a study with 60 cancer patients on cyclophosphamide treatment, showing that individuals carrying an inactive *CYP* allele had significantly decreased cyclophosphamide elimination.(Huang, Roy et al. 2000; Timm, Kaiser et al. 2005) Moreover, the *CYP2D6* genotype is also relevant for cancer patients with respect to the action of the antiemetic drugs such as the 5-HT3 receptor antagonists tropisetron and ondansetron. Lower plasma levels and higher frequency and intensity of vomiting has been demonstrated in patients carrying a higher number of active *CYP2D6* gene copies.(Kaiser, Sezer et al. 2002) Antiemetics use is a standardized part of many chemotherapeutic protocols and it appears that an ultrafast metabolizer phenotype makes the effect of the treatment much less effective, and a dose adjustment for these kind of drugs should be considered.(Tremblay, Kaiser et al. 2003; Candiotti, Birnbach et al. 2005; Ho and Gan 2006)

Beyond the CYP genetic variability effect in drug metabolism, identification of SNPs may be extremely useful in future epidemiologic studies. The *CYP1A1* and *CYP1A2* genes are responsible for the metabolism of numerous environmental toxins, procarcinogens and full carcinogens. Moreover, these

genes are induced by exposure to halogenated hydrocarbons such as 2, 3, 7, 8- tetrachloro-dibenzo-p-dioxin (dioxin) or polycyclic aromatic hydrocarbons (PAH) such as benzo(a)pyrene and 3-methycholanthrene.(Hankinson 1995; Whitlock 1999) Induction is mediated by the aryl hydrocarbons receptor (AHR). The ligand-bound AHR translocates to nucleus, where it dimerizes with the AHR nuclear translocator (ARNT). The heterodimeric AHR-ARNT complex then binds to dioxin responsive element (DRE; -TNGCGTG-) within the gene's promoter region, resulting in the transcriptional activation of nearby target gene expression. The reaction leads to transcriptional activation of gene expression. (Hankinson 1995; Whitlock 1999)

Interestingly, the location of the *CYP1A1* and *CYP1A2* genes is on the same chromosome, in a head-to-head orientation, in many species, such as human, dog, mouse, rat and cow. The distance between the start codons of the two genes in the species mentioned above is between 14 to 24 kbps, with no open reading frame in this region. Thus, it is reasonable to hypothesize that these two genes share common regulatory elements in their promoter region. At least one known DNA response element, the DRE, has been shown be highly conserved among species, and has been located in this *CYP1A1* - *CYP1A2* region in the dog.(Nukaya and Bradfield

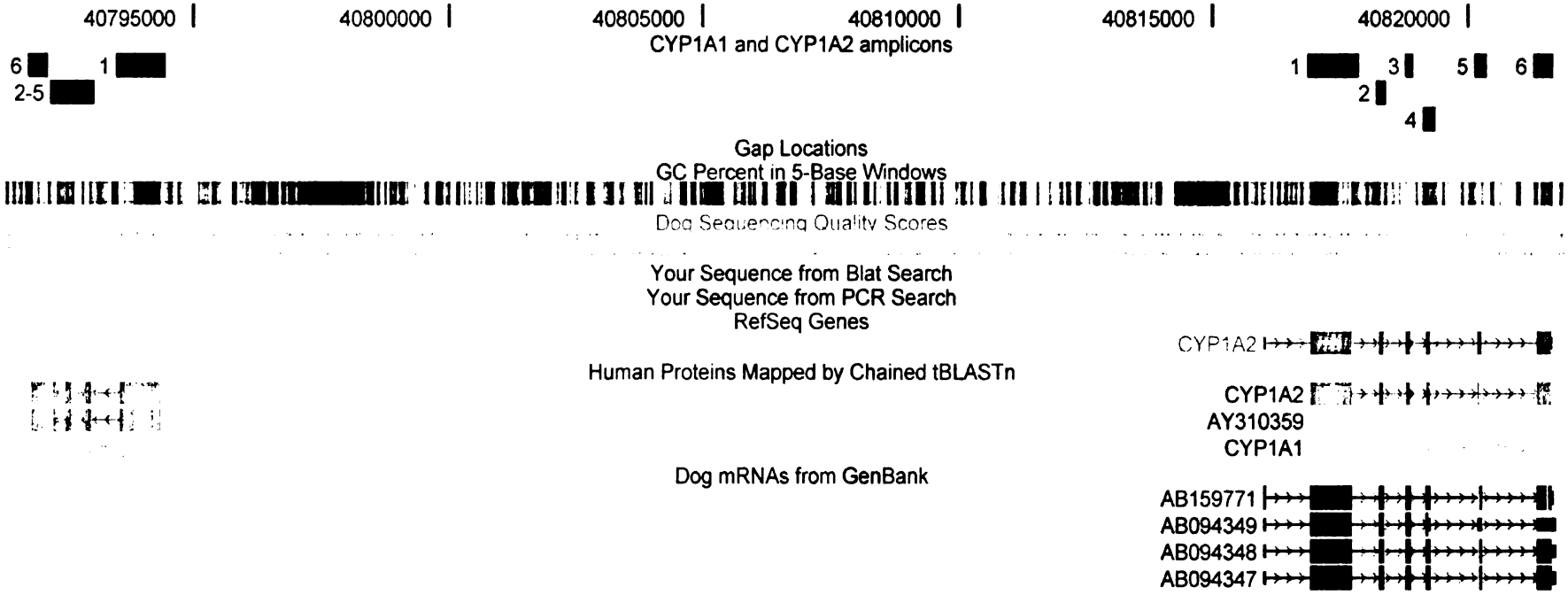
2009) The effect of polymorphic sites in this genomic region remains largely unexplored.

Currently, differences between different dog breeds in specific cancer incidence, increased sensitivity of certain breeds to specific drugs are attributed largely to idiosyncratic reasons.(Mutsaers, Widmer et al. 2003; Modiano, Breen et al. 2005; Dennis, Ehrhart et al. 2006; Rosenberger, Pablo et al. 2007) Our study has demonstrated significant differences in variable alleles between dogs of different breeds, with specific alleles being the major allele in one breed and the minor allele in a different breed. This allelic variability between dogs of different breeds may account to some extend for differences observed in the incidence of carcinogen - induced cancer, and to the sensitivity of certain breeds or individual patients to a specific drug.(Shenton, Chen et al. 2004; Brazzell and Weiss 2006; Mayer, Glos et al. 2008; Vickery, Wilson et al. 2008)

Pharmacogenomics is defined as the study of how genetic variability influences response to administered drugs. A greater understanding of the genetic determinants of drug response has the potential to revolutionize the use of many medications, particularly in the challenging field of oncology. By increasing our ability to prospectively identify patients at risk for severe toxicity, or those

likely to benefit from a particular treatment, pharmacogenomics promises to help us move toward the ultimate goal of individualized cancer therapy. Thus, the need for developing the tools to identify genetic variants, their allele frequency in various dog breeds, and their functional consequences, is of paramount importance.

Figure 11: Genomic region between CYP1A1 and CYP1A2 canine genes on chromosome 30. The CYP1A1 gene is located on the left with orientation for right to left, while the CYP1A2 gene is located on the right, with orientation for left to right. The amplicons for each gene are noted as numbers, with 1 being the first exon of each gene.



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