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EVALUATION OF ANTINEOPLASTIC ACTIVITY OF
ARTEMISININ-DERIVED TRIOXANES IN CANINE TUMORS

(イヌ悪性腫瘍に対するアルテミシニン誘導体トリオキサン
の抗腫瘍効果に関する研究)

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1. Preface

1.1 History of artemisinin in the treatment of human *Plasmodium* infection (malaria)

Artemisinin is a sesquiterpene lactone currently used in the treatment of malaria. Malaria is a fatal infectious disease caused by four species of the blood parasite *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* (Dhinga, 2000). Emergence of multidrug-resistant malaria has been a major threat to public health in Africa and South Asia since the late 1960s (Mackenzie, 1999; Butler, 1997; Riedley, 2002; Guerin, 2002). *Artemisia annua* L. (also known as Qinghaosu, or Sweet wormwood) is a plant belonging to the *Asteraceae* family native to China, which has long been used in traditional Chinese medicine (TCM) for the treatment of chills and fever (Nosten, 1995). In 1967, a program was launched by the Chinese government to screen for antimalarial principles in the various plants used in TCM, which eventually led to the discovery of a highly active chemical extracted from *Artemisia annua* L in 1972. The chemical is now known as artemisinin (also known as qinghaosu) (Qinghaosu Antimalaria Coordinating Research Group, 1979; Jiang, 1982). The parasitocidal effect of

artemisinin and its derivatives were impressive; they induced a rapid arrest of parasite metabolism at concentrations in the low nanomolar range and a more rapid death of parasites than other antimalarial agents (White, 1994). Artemisinin and its derivatives (i.e., artemisinin-derived 1,2,4-trioxanes) cause structural changes in the erythrocyte stage of the parasite by affecting the membranes of the food vacuole, nucleus, mitochondria, endoplasmic reticulum, and nucleoplasm, leading to the formation of autophagous vacuoles and loss of cytoplasm, which kill the parasite (Anon, 1979; Maeno, 1993). Discovery of artemisinin and its derivatives was a milestone in the treatment of malaria due to its remarkable efficacy in curing multidrug-resistant malaria (Li, 1998; Klayman, 1985; Ziffer, 1997; Haynes, 1997), although their use is still restricted by cost in developing countries.

The chemical structure of artemisinin contains a unique endoperoxide trioxane moiety (the bridge-like structure is specifically called an endoperoxide bridge, Figure 1.1). Artemisinin is relatively easily purified by crystallization after extraction from the *Artemisia annua* L. plant from which more lipid- or water-soluble derivatives can be semi-synthesized. However, these endoperoxide-containing trioxanes are extremely difficult to

synthesize *de novo* (Woodrow, 2005). Through a reaction catalyzed by iron, artemisinin-derived trioxanes are converted to carbon-centered free radicals (Meshnick, 1991; Meshnick, 1994). These trioxanes are first activated by intraparasitic heme-iron, which catalyzes the cleavage of the endoperoxide. The release of heme-iron during hemoglobin digestion within the parasite's food vacuole facilitates the cleavage of the endoperoxide moiety by a Fe(II)-Fenton reaction. Breakage of the endoperoxide bridge results in the generation of reactive oxygen species (ROS), such as hydroxyl radicals and superoxide anions (Bergman, 1997; O'Neill, 2004). The parasitocidal effect of artemisinin-derived trioxanes is thought to be mediated by this production of free radicals, because artemisinin derivatives that lack the endoperoxide bridge, a known source of free radicals, do not have antimalarial activity (Brossi, 1988; Klayman, 1985). Antimalarial activities are enhanced by high oxygen tension or other free radical generating compounds (Krungkrai, 1987; Elford, 1987), and free radical scavengers block antimalarial activity (Krungkrai, 1987; Meshnick, 1989). Although this evidence strongly suggests free radical generation is involved in the parasitocidal mechanism, the exceptionally high *in vitro* activities of artemisinin-derived trioxanes

compared to other free radical generating drugs cannot be explained entirely. More recently, alkylation of specific target proteins, rather than ubiquitous oxidation, has been suggested as an alternative mechanism for the marked parasiticidal effect of artemisinin-derived free radicals. The postulated target proteins include the *P. falciparum* translationally controlled tumor protein (TCTP) and the *P. falciparum* orthologue of the calcium ATPase (sarcoplasmic endoplasmic reticulum calcium ATPase, SERCA) (Eckstein-Ludwig, 2003; Asawagahasakda, 1994; Bhisutthibhan, 2001). More definitive mechanism(s) of action remain to be identified.

1.2 Artemisinin-derived trioxanes in cancer therapy

Recently, artemisinin-derived trioxanes have been found to have antineoplastic properties (Woerdenbag, 1993; Efferth, 1996; Efferth, 2001; Singh, 2001; Efferth, 2003; Singh, 2004). The tumoricidal effects are also thought to be mediated by free radical generation. Since iron is a cofactor in the synthesis of deoxyriboses, most neoplastic cells overexpress cell surface transferrin receptors and have greater intracellular iron concentrations than normal somatic cells (May, 1985). For this reason, artemisinins are

selectively cytotoxic for neoplastic cells. Lai et al. (1995), and Singh, et al. (2001) reported that both human leukemia cells and human breast cancer cells are more susceptible to the cytotoxicity of artemisinin than normal human lymphocytes and breast epithelial cells, respectively, by a factor of up to 100. Several additional studies have demonstrated that artemisinin and its derivatives have cytotoxic effects against multiple cancer cell lines *in vitro* (Lai, 2005; Efferth, 2004; Nam, 2007; Pik, 2006; Efferth, 2002; Zheng, 1994; Beekman, 1998; Posner, 1999) and *in vivo* (Moore, 1995). A few case reports have documented the clinical efficacy of artemisinin derivatives in humans with laryngeal squamous cell carcinoma (Singh, 2002), metastatic uveal melanoma (Berer, 2005), and pituitary macroadenoma (Singh, 2006). To our knowledge, no *in vitro* or *in vivo* studies of the potential antineoplastic effects of artemisinin in canine cancer cells have been published.

1.3 Osteosarcoma in dogs and its comparative aspects

Osteosarcoma is the most common primary bone tumor in dogs, accounting for up to 85% of all malignant neoplasm in the bone (Brodey, 1969; Ling, 1974; Lui, 1977; Brodey, 1963; Brodey, 1959). The estimated

incidence of canine osteosarcoma in the United States is approximately 8,000 to 10,000 cases per year (Withrow, 1991; Priester, 1980); however, the true incidence is probably much higher since many pet animals do not have histologic confirmation. Osteosarcoma is primarily a disease of large and giant breed dogs and Greyhounds (Kistler, 1981; Dernell, 2007). Breeds reported to be at an increased risk include the Saint Bernard, Great Dane, Irish Setter, Doberman Pinscher, Rottweiler, German Shepherd Dog, and Golden Retriever (Dernell, 2007). Approximately 75% of canine osteosarcomas occur in the appendicular skeleton and 25% occur in the axial skeleton (Brodey, 1969; Heyman, 1992). For appendicular osteosarcoma, forelimbs are affected twice as often as hindlimbs (Knecht, 1978).

The clinical behavior of canine osteosarcoma is highly aggressive. Although radiographic evidence of metastases is uncommon, approximately 90% of affected dogs develop metastatic disease (Brodey, 1969; Spodnick, 1992). The median survival time after amputation alone is 134 days, with extremely low 1- and 2- year survival rates of 11.5% and 2.0%, respectively (Spodnick, 1992). Thus, although a considerable amount of effort has been made to develop limb-sparing techniques that improve local tumor control

(Vasseur, 1987; Berg, 1992; LaRue, 1989; Thrall, 1990; Withrow, 1993; Straw, 1996), survival times are still limited by the development of metastatic disease. Adjuvant systemic chemotherapy inhibits the progression of metastatic disease and thereby prolongs survival. Numerous studies have evaluated different types of chemotherapy for canine osteosarcoma and all showed a survival advantage compared to amputation alone; however, further improvements in disease-free intervals and overall survival have not been made (Straw, 1991; Bacci, 1988; Shapiro, 1988; Thompson, 1992; Kraegel, 1991; Bergman, 1996; Berg, 1995; Mauldin, 1988; Berg, 1997; Chun, 2000; Kent, 2004; Bailey, 2003; Kirpensteijn, 2002; Withrow, 1995). The general trend in adjuvant chemotherapy for residual micrometastatic disease will likely be towards non-cytotoxic chemotherapeutics, such as molecularly targeted pharmaceuticals, in combination with conventional cytotoxic drugs, rather than further investigation of cytotoxic drug combination.

Spontaneously occurring osteosarcoma in the dog has many similarities to its human counterpart, and has been proposed as a large animal model for human osteosarcoma (Withrow, 1991). The high annual

incidence of canine osteosarcoma (>8,000/year) compared to human osteosarcoma (1,000/year) is particularly attractive for this purpose. The clinical behavior of osteosarcoma in human is as aggressive as canine osteosarcoma; 85-90% of the cases are histologically high-grade (95% in dogs), and the metastatic rate is 80% within 2 years without chemotherapy (90% within 1 year in dogs). The most common metastatic sites are lungs, bone, and soft tissue, which is similar to dogs. As in dogs, improvement in prognosis with adjuvant chemotherapy is observed; however, the long-term survival rate, despite aggressive chemotherapy, is approximately 60%. Further improvement in prognosis with systemic cytotoxic chemotherapy is unlikely without significantly increasing toxicity. Investigation of non-cytotoxic chemotherapy and other strategies in canine osteosarcoma may contribute to the advancement of treatment in human osteosarcoma.

1.4 Altered iron metabolism in canine histiocytic sarcoma

Canine histiocytic sarcoma is a tumor of dendritic cell or macrophage origin (Affolter, 2002). It is further subclassified into ones of

antigen-presenting dendritic cell origin and ones of phagocytic activated macrophage origin; the latter occasionally present with signs of hemolysis and thrombocytopenia known as hemophagocytic syndrome. Clinically, canine histiocytic sarcoma either presents as an isolated lesion or multifocal lesions. The former is termed localized histiocytic sarcoma, often of dendritic cell origin without showing the signs of hemophagocytic syndrome until progressed to advanced stage, and the latter is termed disseminated histiocytic sarcoma, some of which are of macrophage origin and present with marked hemophagocytic syndrome at diagnosis.

Many diseased conditions can cause alteration in iron metabolisms. Ferritin is an iron-binding protein for cells to store iron and is also one of the acute phase proteins. Normally, it is synthesized by the hepatocytes and cells of hematopoietic origin (Yoda, 1980). Hyperferritinemia has been documented in several diseases in human including malignant neoplasia, hemolytic disorders, and liver disease. In particular, hyperferritinemia has been reported with malignant histiocytosis, a human counterpart of canine disseminated histiocytic sarcoma (Esumi, 1989; Ya-You, 1998). Occurrence of hyperferritinemia has been investigated in dogs and has been associated

with liver disease, malignant tumor including histiocytic sarcoma and lymphoma, immune-mediated hemolytic anemia or any other erythrocyte destruction, and marked inflammation (Friedrichs, 2010; Nielsen, 2011). Among these diseases, canine histiocytic sarcoma has been shown to cause marked hyperferritinemia and serum ferritin concentration is proposed to be utilized as an early tumor marker for this malignancy (Nielsen, 2011). Disseminated form of canine histiocytic sarcoma, previously termed as malignant histiocytosis, is known to cause hemolysis and thrombocytopenia, with marked similarity with immune-mediated hemolytic anemia from a clinical standpoint. Together with the documented marked hyperferritinemia, the hemophagocytic characteristic of this tumor leads to the assumption that canine histiocytic sarcoma cells contains higher intracellular iron contents, although this has not been evaluated.

1.5. Aims of the study

In this study, the clinical adverse effects and potential antitumor effects of orally administered artemisinin were investigated in dogs with

spontaneous tumors. To further investigate the mechanism of cytotoxicity, the *in vitro* cytotoxic effect of dihydroartemisinin, an active metabolite of most artemisinin-derivatives, was demonstrated using four canine osteosarcoma cell lines. Dihydroartemisinin induced concentration-dependent free radical generation by an iron-dependent mechanism, resulting in growth inhibition in all four cell lines tested. Dihydroartemisinin also induced cellular apoptosis and G₂/M cell cycle arrest in a concentration-dependent manner. The correlation between the cytotoxicity of dihydroartemisinin and intracellular iron concentration was also investigated in two canine histiocytic sarcoma cell lines. The cytotoxicity of dihydroartemisinin was enhanced by increasing the cellular iron concentration and inhibited by chelating iron from the culture media.

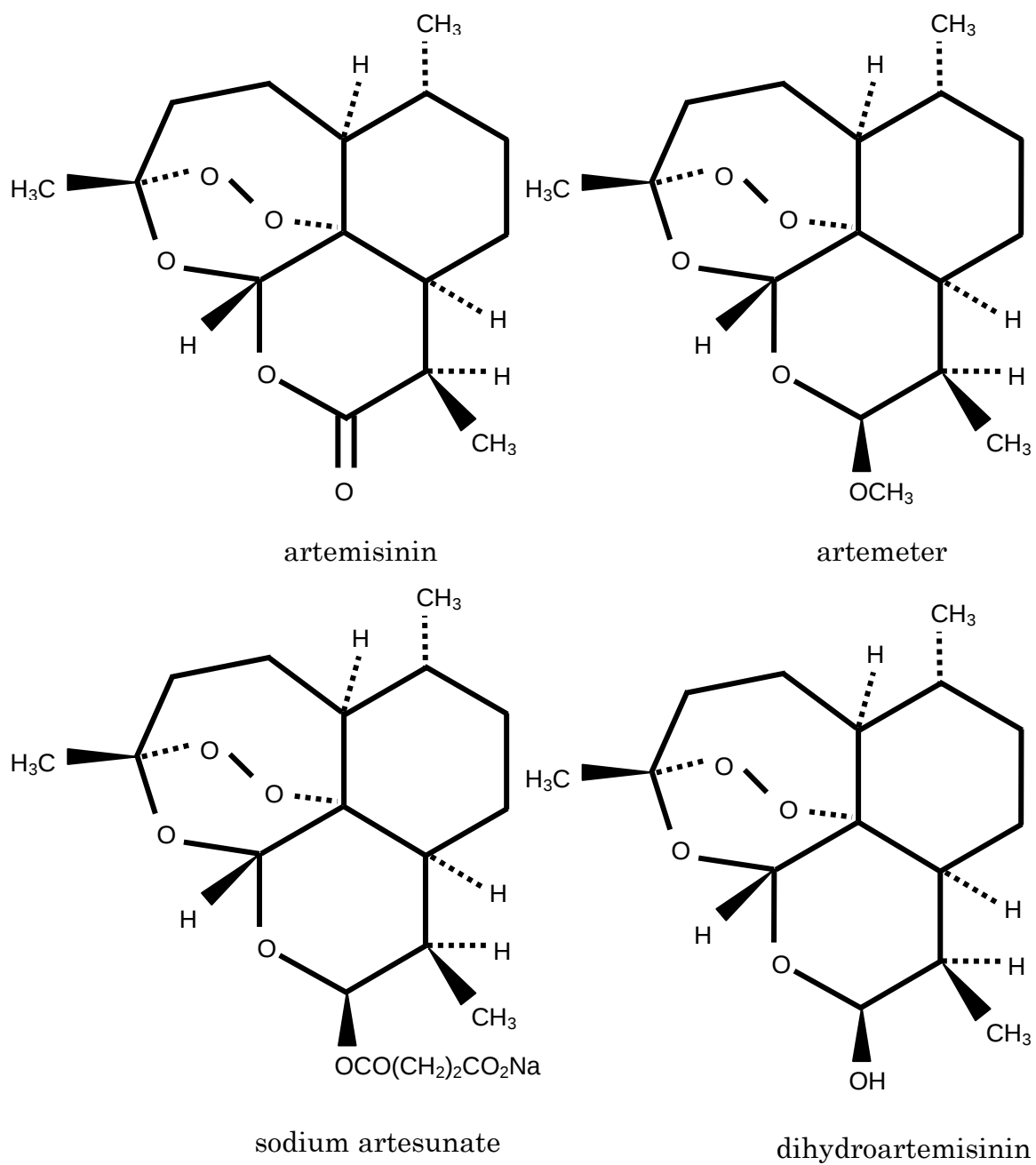


Figure 1.1. Chemical structure of artemisinin-derived 1,2,4-trioxanes.

2. Phase I Clinical Trial of Oral Artemisinin in Cancer-Bearing Dogs: Comparison of High-Dose Intermittent and Low-Dose Continuous Dosing Schedules in Dogs with Naturally Occurring Tumors

2.1. Summary

To evaluate the clinical toxicity and activity of orally administered artemisinin in dogs with spontaneous tumors, 24 client-owned dogs were randomly divided into two groups and received either low-continuous dose (3 mg/kg, q24h) or intermittent-high dose (3 doses of 45 mg/kg, q6h, repeated once weekly) of artemisinin orally. Treatment was continued for 21 days. Dogs were evaluated weekly for clinical, hematological, and biochemical adverse events. Whole blood concentrations of artemisinin and dihydroartemisinin were measured by liquid chromatography/tandem mass spectrometry after the first dose of artemisinin in three dogs in each group. All dogs tolerated oral artemisinin in both groups. The most frequent adverse event was anorexia, which was observed in 11% of the low dose group and 29% of the high dose group. Objective tumor response was observed in one dog with a urinary bladder transitional carcinoma, which

lasted for 6 weeks. Blood concentrations of artemisinin and dihydroartemisinin were below 0.1 μM at all time points, and there was no difference in blood concentration between the two dosing groups. Oral artemisinin, both in low- continuous dose and high- intermittent dose, is well-tolerated in dogs but results in low bioavailability. The parenteral administration route should be considered for future studies.

2.2. Introduction

Artemisinin is a sesquiterpene lactone extracted from a plant *Artemisia annua L.*, which is used in TCM. It was first identified and isolated in 1972 in a project to discover new antimalarial drugs from TCM launched by the Chinese government (Qinghaosu Antimalaria Coordinating Research Group, 1979; Klayman, 1985), and now is the first line treatment of malaria in countries in South Asia (Li, 1994; Hien, 1994).

Artemisinin has a unique chemical structure, an endoperoxide bridge; by a cleavage of the endoperoxide bridge catalyzed by iron, artemisinin becomes a carbon-centered free radical (Meshnick, 1993; Zhang, 1992). This reactive free radical results in damage to lysosomal membranes, leading to autodigestion (Bergman, 1997; O'Neill, 2004), and alkylation of essential proteins of malaria parasites, including *Plasmodium falciparum* translationally controlled tumor protein (TCTP) (Asawamahasakda, 1994; Bhisutthibhan, 2001; Eckstein-Ludwig, 2003).

More recently, artemisinins have been found to have antineoplastic properties (Woerdenbag, 1993; Efferth, 1996; Efferth, 2001; Singh, 2001; Efferth, 2003; Singh, 2004). Tumor cells often overexpress transferrin

receptors to uptake iron, which is a cofactor of deoxyribose synthesis; thus, they have higher intracellular iron concentrations than their somatic counterparts (Aulbert, 1980; Karin, 1981; Reizenstein, 1991; Raaf, 1993; Shterman, 1991; Castaneda, 1991; Das-Gupta, 1996). The mechanism of artemisinin's anticancer activity is also thought to be through generation of free radicals mediated by intracellular iron molecules. The cellular target of these free radicals have not been completely identified, but the expression level of TCTP correlates with sensitivity to artemisinin derivatives, suggesting that TCTP may be one of the target proteins in anticancer mechanism (Efferth, 2005); however, other cellular structures, including mitochondrial membranes and DNA can also be damaged. Alternatively, some of the antitumor effects of artemisinin may not be due to direct cytotoxic effect, but rather due to indirect effects, such as inhibition of neoangiogenesis (Zhou, 2007; Ricci, 2010) or modification of the T-regulatory response (Langroudi, 2010; Noori, 2009).

Several studies have demonstrated that artemisinin and its derivatives have cytotoxic effects against multiple human cancer cell lines *in vitro* and against a rat fibrosarcoma cell line *in vivo* (Woerdenbag, 1993;

Efferth, 1996; Efferth, 2001; Singh, 2001; Efferth, 2003; Singh, 2004; Lai, 1995; Lai, 2005; Efferth, 2004; Nam, 2007; Pik, 2006; Efferth, 2002; Zheng, 1994; Beekman, 1998; Posner, 1999; Moore, 1995). To date, three case reports have been published in human medicine documenting tumor control by artesunate in laryngeal squamous cell carcinoma, metastatic uveal melanoma, and pituitary macroadenoma (Singh, 2002; Berger, 2005; Singh, 2006). In veterinary medicine, there is a case report of a dog with an appendicular osteosarcoma that had a complete response to oral artemisinin (Rowley, 2004); however, this report was not published in a peer-reviewed journal.

Despite long-term use of artemisinin for the treatment of malaria, there is a paucity of information on the pharmacokinetics of artemisinin and its derivatives. The dosages and administration schedules of artemisinin derivatives in both veterinary and human medicine are mostly anecdotal. Orally administered artemisinin is commonly used in veterinary medicine, with low-dose (100mg/dog) daily or twice daily administration. In general, after oral administration, artemisinin and its derivatives are rapidly absorbed from the gastrointestinal tract, with peak plasma concentration

occurring in one hour (Dhingra, 2000). All artemisinin derivatives except artemisinin *per se* (plasma metabolite of artemisinin is currently unknown [Navaratnam, 2000]) are primarily metabolized in the liver to the active metabolite dihydroartemisinin (DHA), and then eliminated in urine and feces in a relatively short period, with a plasma half-life ranging from 45 minutes to 11 hours (Dhingra, 2000). Pharmacokinetics of artemisinin is significantly influenced by individual variation, gender, and concurrent fat intake (Navaratnam, 2000). There also is a significant decrease (oral and rectal administration) or increase (intramuscular administration) in the area under the curve with repeated administrations, which is of great concern since most pharmacokinetic studies were performed after administering a single dose. After daily oral administration of artemisinin, the AUC at day 7 is only 24% of that of day 1 in healthy adults (Ashton, 1998). Similar results were obtained in dogs (Classen, 1999). This suggests that the administration schedule of artemisinin commonly used in dogs (daily or twice daily oral administration) may not be clinically effective because most of the administered artemisinin may not be absorbed.

To our knowledge, no studies investigating the safety and

antineoplastic effects of artemisinin in dogs with cancer in a clinical setting have been published. Therefore, this study was conducted to evaluate the *in vivo* effects of oral artemisinin, the most commonly used over-the-counter artemisinin derivatives, in dogs with spontaneous cancers.

2.3. Materials and methods

2.3.1. Animals

Twenty-four client-owned dogs with various spontaneous tumors were included in the study. The inclusion criteria included; measurable tumor burden, histopathologic or cytopathologic confirmation of the tumor type, failure of conventional treatments or the owner's consent to use artemisinin in lieu of conventional therapy, expected survival time of >4 weeks, and written owner's consent. The study protocol was approved by the Veterinary Teaching Hospital Board. The exclusion criteria included concurrent severe renal or hepatic disease and concurrent use of therapies other than analgesics. The use of non-steroidal anti-inflammatory drugs was permitted only if they were necessary for analgesic purposes, and they had been initiated >4 weeks prior to enrollment into the current study and the patient had no measurable tumor response. Two additional healthy dogs owned by hospital staff were used for a pharmacokinetic analysis of the oral artemisinin, as insufficient number of the clients agreed to hospitalize their dogs to obtain multiple blood samples.

2.3.2. Artemisinin

Artemisinin with greater than 99% purity was purchased from a commercial supplier (Holleypharma, Tustin, CA). The drug was available as either 100- or 50-mg capsules, which were reformulated to produce smaller capsules when required by the standard dosage used by the study. The capsules were administered orally in conjunction with fat-containing food (peanut butter) for pilling purpose and to maximize the intestinal absorption of the artemisinin.

2.3.3. Treatment schedule

Dogs were randomly assigned to either Group 1 (low-continuous dose; 3 mg/kg, PO, q24h) or Group 2 (high-intermittent dose; 45 mg/kg, PO, q6h for 3 doses, repeated once weekly). Absorption of orally administered artemisinin can be affected by the fasting status. For the pilling purpose and to minimize variability of intestinal absorption, artemisinin capsules were always given with a same amount of fat containing food (i.e., peanut butter). Dogs were fully evaluated on the day of the enrollment, by means of a

complete physical examination, complete blood count (CBC), serum biochemical profile, urinalysis, and appropriate imaging modality (radiographs or ultrasonography) when tumor measurement required such imaging techniques. The dogs were readmitted and physical examination and gross tumor measurement were repeated once weekly or whenever the attending clinician judged reassessment was necessary. On week 4, a complete physical examination, CBC, serum biochemical profile, urinalysis, and tumor imaging, when necessary, were repeated to make a final assessment of tumor response and potential adverse effects. The owners were given a standardized adverse event assessment scheme, and asked to document any changes/abnormality of their dogs that could potentially be a drug-related adverse effect. The owner's record was evaluated by one of the investigators at each visit, and adverse effects were graded according to the Veterinary Cooperative Oncology Group Common Terminology for Criteria of Adverse Event (Veterinary Cooperative Oncology Group, 2004).

2.3.4. Pharmacokinetic study

Blood samples were collected from one dog from Group 1 and three

dogs from Group 2 for pharmacokinetic analysis of artemisinin and its putative metabolite, dihydroartemisinin. Two additional clinically healthy dogs, owned by hospital staff, were given one dose of 3 mg/kg of artemisinin. For the dogs receiving conventional doses (one dog in Group 1 and two healthy volunteers), samples were collected at 5, 10, 20, 35, 60, and 90 minutes and at 2, 4, 6, 9, 12, and 16 hours after drug administration on day 1. For Group 2, time points for blood collection included immediately before the first, second, and third doses and at 5, 10, 20, 35, 60, and 90 minutes and at 2, 4, 6, 9, 12 and 16 hours after the third dose was administered on day 1.

Artemisinin derivatives have been shown to bind significantly to red blood cells (Li, 1998). A modified procedures reported by Naik, et al. (2005) utilizing liquid chromatography/tandem mass spectrometry (LC/MS/MS) analytical method with atmospheric pressure chemical ionization (APCI) methods was used for determination of whole blood concentrations of both artemisinin and dihydroartemisinin, which was validated to measure artemisinin and dihydroartemisinin within canine whole blood samples at the investigators' laboratory.

2.4. Results

2.4.1. Patient population

Twenty-four dogs were included in the study; 12 dogs received low-continuous dose (Group 1), and 12 received intermittent-high dose (Group 2). The tumor type included metastatic/unresectable osteosarcoma (n=7), transitional cell carcinoma of the urinary bladder (n=5), soft tissue sarcoma (n=3), mammary gland carcinoma (n=2), malignant melanoma (n=2), and each one of multicentric lymphoma, chondrosarcoma, mast cell tumor, hemangiosarcoma, and invasive adrenal gland tumor. Most of these dogs had advanced stage disease, and eight dogs did not complete the planned treatment course for various reasons; three dogs died or were euthanized before completion of the study due to tumor progression, two dogs did not return for recheck for unknown reason, the owner of another dog decided to start chemotherapy before completing the study, artemisinin treatment was not given as instructed in one dog, one dog became too aggressive to pill and oral artemisinin was discontinued. Sixteen dogs (9 dogs in Group 1, 7 dogs in Group 2; Table 1) completed the protocol and were available for toxicity and tumor response evaluation.

2.4.2. Hematological toxicities

No significant changes in hematocrit ($p=0.30$), segmented neutrophil count ($p=0.66$), or platelet count ($p=0.71$) were seen. Grade 1 neutropenia ($1.9 \times 10^9/L$ and $2.8 \times 10^9/L$, respectively) was seen in 2 dogs (Dog #1 and #4, both in the Conventional group); however, the pretreatment neutrophil counts in these dogs were low ($2.8 \times 10^9/L$ and $3.1 \times 10^9/L$, respectively). These neutrophil counts were interpreted as physiologic values for Dog #1 (a 10 year-old Greyhound). The reason for mild neutropenia in Dog #4 was unknown, but thought to be unrelated to artemisinin administration. Grade 1 anemia was seen in 2 dogs (Dog #10 and #5). No thrombocytopenia was observed.

2.4.3. Biochemical toxicities

There were 2 dogs with BUN increase over baseline; in Dog #1, BUN increased from 20 mg/dL to 27 mg/dL; and in Dog #8, it increased from 16 mg/dL to 33 mg/dL. Neither of these 2 dogs had relevant changes in serum creatinine concentration over baseline; no dogs had significant increases in

serum creatinine concentration. Two dogs had elevations of serum ALT activity over baseline; Dog #1 had ALT activity increase from 51 IU/L to 58 IU/L; Dog #16 had increase of serum ALT activity from 85 IU/L to 125 IU/L. Both were considered to be clinically irrelevant.

2.4.4. Clinical adverse events

Potential neurotoxicity was seen in 1/16 dogs (6%). This dog (Dog #13) developed tremors after the first dosing of high-dose artemisinin; however, the dog was diagnosed with a urinary tract infection by the referring veterinarian, and the tremors resolved after initiation of antibiotics. The dog did not have any adverse event after the subsequent doses of high-dose artemisinin.

Grade 2 lethargy was seen in 2 dogs (Dog #5 and #13). As discussed above, dog #13 was diagnosed with urinary tract infection, and the lethargy resolved after initiation of antibiotics.

Anorexia was seen in 3 dogs (Dog #10 [Grade 1], #5 [Grade 1], #13 [Grade 2]). Dog #10 developed Grade 1 anorexia after the first high-dose artemisinin, but no such adverse event was seen after the subsequent doses.

Dog #13 was diagnosed with urinary tract infection, and the anorexia resolved after initiation of antibiotics. Vomiting was seen in 1 dog (Dog #2, Grade 1). The dog had an episode of vomiting after the first dose of low-dose artemisinin, but none after the subsequent doses. Diarrhea was seen in 2 dogs (Dog #5 [Grade1], Dog #13 [Grade 2]). Dog #13 was diagnosed with urinary tract infection, and the diarrhea resolved after initiation of antibiotics.

2.4.5. Tumor response

Nine dogs completed the treatment protocol in Group 1 and 7 dogs in Group 2. In Group 1, 3 dogs had PD and 5 had SD. In Group 2, 1 dog had PR (TCC, Fig 1a and 1b), 4 had PD, and 2 had SD. In the dog that had PR, the treatment was continued for 3 additional weeks, and disease progression was noted 6 weeks from the start of the treatment.

2.4.6. Whole blood concentration measurement

The measured whole blood concentrations of artemisinin and dihydroartemisinin were less than 0.1 μ M at all points after administration

of conventional doses of artemisinin. No meaningful pharmacokinetic analysis was possible due to the extremely low blood concentration of the agent. This low level of blood concentration was not improved by administration of high-dose artemisinin at short intervals; no significant increase in blood concentrations of artemisinin or dihydroartemisinin was observed with IHD group, suggesting poor and saturable gastrointestinal absorption of the drug in dogs.

Table 2.1. Patient characteristics in Group 1 and 2.

Group 1 (3 mg/kg, q24h, continuously)							
Cas e #	Breed	Age	Sex	Weight	Tumor type	Previous therapy	Response
1	Greyhound	10	MC	33.5	OSA	Doxorubicin	PD
2	Boxer	11	FS	25.3	OSA	Doxorubicin	SD
3	Golden	12	MC	34.7	CSA	Radiation therapy	PD
4	Great Dane	3	MC	61.3	STS	None	SD
5	Mix	9	FS	24.4	MM	None	SD
6	Mix	12	MC	13.8	TCC	Surgery	PD
7	Greyhound				Adrenal	None	SD
8	Mix				TCC	None	SD
9	Shelty				STS	None	SD
Group 2 (45 mg/kg, q6h for 3 doses, once weekly)							
Case #	Breed	Age	Sex	Weight	Tumor type	Previous therapy	Response
10	Mix	6	FS	48.0	OSA	Doxorubicin	PD
11	Mix	13	FS	11.5	TCC	CBDCA/Gem	PR
12	Mix	6	FS	39.1	MCT	CCNU/VBL	PD
13	Samoyed	9	FS	26.3	MGT	Surgery	PD
14	Mix	5	FS	36.9	OSA	Doxorubicin	PD
15	Golden	12	FS	28.5	MGT	CBDCA	SD
16	Mix	12	FS	10.5	TCC	Mitoxantrone	SD

OSA: osteosarcoma, CSA: chondrosarcoma, STS: soft tissue sarcoma, MM: malignant melanoma, TCC: transitional cell carcinoma, MGT: mammary gland tumor.

PR: partial response, SD: stable disease, PD: progressive disease.

Table 2.2. Gastrointestinal toxicities of oral artemisinin in dogs. All toxicities were grade 1 or 2, and no grade 3 toxicity was observed.

Toxicity	Overall	Group 1	Group 2
Lethargy	13%	11%	14%
Anorexia	19%	11%	29%
Vomiting	6%	11%	0%
Diarrhea	13%	11%	14%

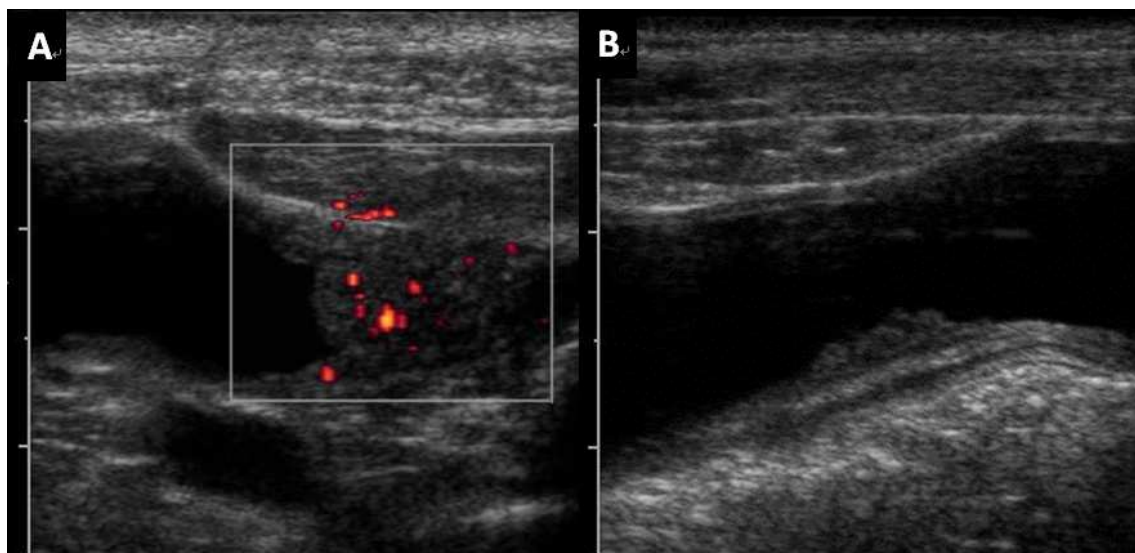


Figure 2.1. Ultrasonographic images of the urinary bladder in Dog 11 on Day 1 (A) and Day 22 (B). A vascularized tumor protruding in to the lumen became flattened and less obvious after 3-week course of treatment.

2.5. Discussion

During the past two decades, the anti-malarial agent artemisinin and its derivatives have attracted significant interest as potential novel anticancer agents (Woerdenbag, 1993; Efferth, 1996; Efferth, 2001; Singh, 2001; Efferth, 2003; Singh, 2004; Lai, 1995; Lai, 2005; Efferth, 2004; Nam, 2007; Pik, 2006; Efferth, 2002; Zheng, 1994; Beekman, 1998; Posner, 1999; Moore, 1995), cancer preventatives (Lai, 2006; Disbrow, 2005), multidrug resistance reversal agents (Mukanganyama, 2002; Reungpatthanaphong, 2002), and radiosensitizers (Kim, 2006). These artemisinin-derived 1,2,4-trioxanes exhibit significant activity in the nanomolar to micromolar range against a variety of human cancer cell lines. The biologic activity in human cancer cell lines and apparently low toxicity in human malaria patients have stimulated the use of artemisinin by dog owners to treat various canine malignancies, particularly osteosarcomas, as the drug is readily available without a prescription. However, to our knowledge, there are no reports on the safety and potential activity of artemisinin in dogs with cancer *in vivo*.

The prevalence of hematological/biochemical toxicity in the current

study was low. The Grade 1 anemia seen in 2 dogs were likely secondary to disease progression (i.e.; anemia of chronic disease) rather than to artemisinin toxicity. The Grade 1 neutropenia seen in 2 dogs were interpreted as normal value for these individuals. Similarly, the 2 events of high serum BUN concentration (both in Group 1) were interpreted as secondary to disease progression and dehydration, or subclinical gastrointestinal bleeding, as none of the dogs developed increases in serum creatinine concentration. An unreported change in diet by the owners to one with higher protein content may also have accounted for the increases in BUN in light of normal creatinine concentrations. Two dogs had minimal increases in the serum ALT activity; these minor changes were thought to be clinically irrelevant and likely a result of daily fluctuation of ALT values. However, the possibility of hepatotoxicity associated with high-dose artemisinin could not be ruled out, as Dog #19 developed Grade 2 ALT increase.

This study also shows that the neurotoxicity reported in rodents studies are not common in dogs at the dosing scheme used in this study. The only questionable neurotoxicity seen in the study population was manifested

by tremors in 1 dog; however, the tremors resolved with the treatment of urinary tract infection. Since the same effect was not observed after the subsequent three weekly treatments, we believe this was not treatment-related.

Both low-dose continuous and high-dose intermittent regimens were well-tolerated. No dogs developed Grade 3 or higher toxicities. Occasional anorexia and vomiting were seen in small number of dogs, but they were self-limiting and clinically acceptable.

No durable tumor response was observed in the study; objective response was observed only in one dog, which lasted for a relatively short period of time (6 weeks). This lack of tumor response can be explained by the low blood concentration achieved after oral administration of artemisinin. The blood concentration of artemisinin was well below the therapeutic target concentration (10 μ M, based on our previously reported *in vitro* study [Hosoya, 2008]). The concentration of dihydroartemisinin was even lower. Furthermore, the blood concentrations of artemisinin and dihydroartemisinin were similarly low after administration of high-dose artemisinin. These findings were consistent with the previous study, in which orally

administered artemether (600 mg/kg) resulted in less than 1 nM peak plasma concentration of artemether and less than 0.5 nM peak concentration of dihydroartemisinin (Classen, 1999). This suggests that the bioavailability of orally administered artemisinin is poor and gastrointestinal absorption saturable in dogs, and the conversion of artemisinin to dihydroartemisinin is incomplete. We are currently performing a more detailed pharmacokinetic assay of orally administered and parentally administered artemisinin derivatives in normal dogs.

Although the study showed minimal and acceptable adverse events, it does not support the use of single agent oral artemisinin in the treatment of neoplasia in dogs, as the study showed the targeted therapeutic concentration is not achievable even with high oral dose. Possible ways to circumvent this problem are use of parental formulation and/or use of more potent artemisinin-derivatives, such as artesunate, dihydroartemisinin, or investigational newer generation artemisinin-derivatives.

3. Biologic Activity of Dihydroartemisinin in Canine Osteosarcoma Cell Lines: Effects on Cellular Proliferation, Cell Cycle Distribution, Induction of Apoptosis, and Intracellular Free Radical Contents

3.1. Summary

Artemisinin is a chemical extracted from a plant *Artemisia annua*. It has recently been discovered to have anti-neoplastic properties. It is anecdotally being used by veterinarians and pet owners to treat various type of cancer, especially osteosarcoma. However, evidence is lacking to support its biologic activity against canine osteosarcomas. Spontaneously occurring osteosarcoma in the dog is also an excellent large animal model for human osteosarcoma. In this study, the *in vitro* cytotoxic effect of dihydroartemisinin, a newly emerging potential anti-cancer agent with low systemic toxicity, was investigated in four canine osteosarcoma cell lines. Dihydroartemisinin induced concentration-dependent free radical generation by an iron-dependent mechanism, resulting in growth inhibition in all four cell lines tested. Dihydroartemisinin also induced cellular apoptosis and decrease of G₀/G₁ cell population in a concentration-dependent

manner. This study is the first to demonstrate *in vitro* cytotoxic activity of an artemisinin derivative in canine osteosarcoma and provides relevant information in order to design a clinical trial in canine osteosarcoma.

3.2. Introduction

Artemisinin is an extract from the Chinese plant quinghao (*Artemisia annua L.*) and is used in traditional Chinese medicine for chills and fever (Nosten, 1995). Since 1972, when its antimalarial properties were discovered, artemisinin and its semisynthetic derivatives (i.e., arteether, artemether, artesunate, artelinate, and arteminol [dihydroartemisinin]) have been studied extensively as anti-malarial agents (Jiang, 1982). These compounds react with intracellular iron in free heme molecules within malaria-infected red blood cells and are converted to highly reactive free radicals. Their anti-malarial activity is due to their generation of reactive oxygen species (ROS), which are thought to alkylate one or more essential malarial proteins (Posner, 2000; Meshnick, 1998).

More recently, artemisinins have been found to have antineoplastic properties (Woerdenbag, 1993; Efferth, 1996; Efferth, 2001; Singh, 2001; Efferth, 2003; Singh, 2004). Similar to its anti-malarial effects, the tumoricidal mechanism of artemisinin is secondary to ROS generation. Since iron is a cofactor in the synthesis of deoxyriboses, most neoplastic cells

overexpress cell surface transferrin receptors (TfR) and have greater intracellular iron concentrations than normal somatic cells (May, 1985). This may explain the basis of the selective cytotoxicity of artemisinin for neoplastic cells. For example, it has been shown that human leukemia and breast cancer cells are more susceptible to the cytotoxic effects of artemisinin than normal human lymphocytes and breast epithelial cells by a factor of up to 100 (Singh, 2001; Lai, 1995). Several additional studies have demonstrated that artemisinin and its derivatives have cytotoxic effects against multiple human cell lines *in vitro* and against a rat fibrosarcoma cell line *in vivo* (Woerdenbag, 1993; Efferth, 1996; Efferth, 2001; Singh, 2001; Efferth, 2003; Singh, 2004; Lai, 1995; Lai, 2005; Efferth, 2004; Nam, 2007; Pik, 2006; Efferth, 2002; Zheng, 1994; Beekman, 1998; Posner, 1999; Moore, 1995). Finally, the potential clinical efficacy of artemisinin derivatives has been documented in case reports of humans with laryngeal squamous cell carcinoma, metastatic uveal melanoma, and pituitary macroadenoma (Singh, 2002; Berger, 2005; Singh, 2006). Together, these data suggest that artemisinin may be a useful agent to treat cancer in the clinical setting.

Given the low toxicity and antineoplastic activity of artemisinin in

human cancers and cell lines, and anecdotal reports of efficacy in canine cancers, especially osteosarcoma, an increasing number of pet owners are treating their cancer affected dogs with artemisinin, which is readily accessible as an over-the-counter product. To our knowledge, no studies investigating the potential antineoplastic effects of artemisinin in canine cancer cells have been published. Therefore, this study was initiated to evaluate the *in vitro* effects of dihydroartemisinin (DHA), the active metabolite of most artemisinin derivatives, on cell viability, cytotoxicity, and cell cycle progression in canine osteosarcoma cell lines.

3.3. Materials and methods

3.3.1. Cell culture and cell lines

Four canine osteosarcoma cell lines (D17, OSCA2, OSCA16, and OSCA50) were evaluated (OSCA cell lines were kindly provided by Dr. Jaime F. Modiano). The D17 cell line was cultured in RPMI-1640^a supplemented with 10% fetal bovine serum^b (FBS) and antibiotics (penicillin [100 U/ml] and streptomycin [0.1 mg/ml])^c. The other three cell lines were cultured in DMEM supplemented with 10% FBS and antibiotics. All cell lines were maintained in a humidified environment at 37° C under 5% CO₂ and 95% room air.

2.1.2 Chemicals

Dihydroartemisinin (DHA)^e was dissolved in 100% dimethylsulfoxide (DMSO) at the concentration of 100 mM as a stock solution, protected from light, and kept at -80° C for long-term storage. The stock solution was further diluted with DMSO prior to use in tissue culture, resulting in a consistent DMSO concentration of 0.1% volume. DHA solutions were newly

prepared from the stock solution for each experiment.

2.1.3 Assessment of Cell Viability

Cells were seeded in 96-well plates at densities of 1,000–2,000 cells per well in six replicates to a final volume of 100 μ L/well. After 24 hours, 100 μ L of medium containing DMSO (control) or different concentrations of DHA was added to each well to create the final DHA concentrations of 0.1, 1, 5, 10, 50, and 100 μ M and the cells were incubated for additional 48 hours. After treatment, the medium was removed by gentle suction and plates were stored at -80° C until use. Cell proliferation was assessed using the CyQUANT™ nucleic acid fluorescence assay kit^f, according to the manufacturer's instructions. The intensity of fluorescence of the reaction product, after background subtraction, was measured with excitation at 485 nm and emission detection at 530 nm on a Spectra Max M2 plate reader.^g Cell proliferation was expressed as a percentage of the control wells: fluorescence of sample/ fluorescence of control (DMSO only) cells. Experiments were repeated at least three times.

2.1.4 Nucleosome Fragmentation (Cell Death) ELISA

To assess drug-induced apoptotic cell death a Cell Death Detection ELISA kit^h was used according to the manufacturer's instructions. The assay is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes or oligonucleosomes generated after apoptotic cell death. Briefly, $2.0\text{--}5.0 \times 10^5$ osteosarcoma cells were cultured in medium in 6-well plates for 24 hours before treatment. Cells were treated with varying concentrations of DHA (0 to $40 \mu\text{M}$) or DMSO vehicle as a control for 24 hours. Cells were collected and 1.0×10^5 cells were counted and used in the ELISA.

2.1.5 Immunoblotting

Osteosarcoma cell lines were treated with varying concentrations of DHA or DMSO vehicle control and collected after 24 hours of exposure. Cells were lysed in M-PER protein extraction reagentⁱ unless otherwise stated. Additionally, HaltTM Protease inhibitor^j cocktail mix and phosphatase inhibitors^k were added to the lysis buffer. Cells were washed with ice-cold PBS, and resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.0),

137 mM NaCl, 1 mM CaCl₂, 10% glycerol, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin at 10 μg/ml, and aprotinin at 10 μg/ml. Following protein quantitation, equivalent amounts of proteins (50-100 μg) from each lysate were resolved on 8% SDS-polyacrylamide gels^l and transferred to nitrocellulose membranes^m. Membranes were washed with Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBST), blocked in 5% skim milk in TBST for 1 hour at room temperature, and probed with antibodies specific for caspase 3ⁿ, or β-actin^o all at a dilution of 1:500 in TBST/5% BSA overnight at 4° C. The membranes were washed and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase^p at 1:15,000 in 2.5% milk in TBST for 1 hour. Blots were then developed using ECL reagent^q.

2.1.6 Cell Cycle Analysis

For the detection of cell cycle progression and cell death, cell lines were seeded in 6-well plates at a density of 2.0×10^5 cells/well. Cells were incubated for 24 hours, after which the medium was replaced with medium containing DMSO (control) or 1, 10, or 50 μM of DHA. Following 24, 48, or 72

hours of drug exposure, cells were collected, washed with PBS, and stored in 70% ethanol. Cell suspensions were stored at 4° C overnight. Fixed cells were washed in 5 ml of PBS, suspended in 500 µl of PBS containing 100 µg/ml of RNase A and 50 µg/ml of propidium iodide^r. Flow cytometric analysis was performed using a BD FACSCalibur^s instrument. Data were analyzed with Cell Quest Pro software^t. The accumulation of subG₀/G₁ cells, an indicator of DNA fragmentation and apoptosis, was used to quantify cell death. Experiments were repeated twice.

2.1.7 Measurement of Reactive Oxygen Species

Production of ROS was determined using 6-carboxy-2',7'-dichlorodihydrofluorescein^u and flow cytometric analysis as described (16, 17). Briefly, D17 cells (5.0 x 10⁵) were seeded in 50-mm cell culture dishes. Twenty-four hours later, the cells were incubated with PBS (0.1% volume) or with desferrioxamine^v at the concentration of 150 µM. Six hours later, cells were incubated with 10, 25, 50, 100 µM of DHA or DMSO (0.1% volume, control) for 12 hours. The cells were then incubated for 1 hour with 6-carboxy-2',7'-dichlorodihydrofluorescein at the concentration of 10 µM in

RPMI and simultaneously treated with DHA or DMSO (control). The media was removed, cells were rinsed with PBS, then incubated in fresh RPMI containing DHA or DMSO for 3 hours. Cells were collected, resuspended in PBS, and analyzed by flow cytometry. Experiments were repeated twice.

2.1.8 Statistical Analysis

Changes in cell viability, measured by CyQUANT™ assay, and cytoplasmic nucleosome, measured by ELISA, were compared using one-way analysis of variance. A pairwise multiple comparison procedure was performed using the Dunnet test. The proportions of cells in sub-G₀/G₁, G₀/G₁, S, and G₂/M phases of the cell cycle in the control and treated groups at different time points were compared statistically with contingency tables using the Chi-square test. All statistical analysis was performed using GraphPad Prism 4.0 software^w. Statistical significance was established as $P \leq 0.05$.

2.1.9. Footnotes

^a RPMI-1640, GIBCO®, Invitrogen, Carlsbad, CA

- ^b FBS, Gemini, West Sacramento, CA
- ^c Penstrep, GIBCO[®], Invitrogen, Carlsbad, CA
- ^d DMEM, GIBCO[®], Invitrogen, Carlsbad, CA
- ^e Dihydroartemisinin, Sigma, St. Louis, MO
- ^f CyQUANT[™] nucleic acid fluorescence assay kit, Molecular Probes, Leiden, the Netherlands
- ^g Spectra Max M2 plate reader, Molecular Devices, Sunnyvale, CA
- ^h Cell Death Detection ELISA^d kit, Roche Diagnostics Corporation, Indianapolis, IN
- ⁱ M-PER protein extraction reagent, Pierce Biotechnology, Rockford, IL
- ^j Halt[™] protease inhibitor, Pierce Biotechnology, Rockford, IL
- ^k Phosphatase inhibitor, Vanadate, NaF
- ^l Gene Mate Express Gels, ISC-Bioexpress, Keysville, UT
- ^m Biotrace[™] NT, Pall Corporation, Pensacola, FL
- ⁿ Anti-caspase 3 antibody, Cell Signaling Technology Inc., Beverly, MA
- ^o Anti-actin antibody, Sigma, Saint Louis, MO
- ^p Goat anti-rabbit IgG conjugated to horseradish peroxidase, Cell Signaling Technology Inc., Beverly, MA

^q ECL reagent, Perkin-Elmer, Boston, MA

^r Propidium iodide, Sigma-Aldrich, St. Louis, MO

^s BD FACSCalibur, Becton Dickinson, San Jose, CA

^t Cell Quest Pro software, Becton Dickinson-Biosciences, San Jose, CA

^u Aminophenyl fluorescein (APF) and hydroxyphenyl fluorescein (HPF),
Molecular Probes, Eugene, OR

^v Desferrioxamine mesylate, Hospira, Lake Forest, IL

^w GraphPad Prism 4.0, GraphPad Software Inc., San Diego, CA

3.4. Results

3.4.1. Inhibitory effects of dihydroartemisinin on proliferation of canine osteosarcoma cells

To test whether DHA can inhibit canine osteosarcoma cell growth, we determined the growth inhibitory effect of DHA in four canine osteosarcoma cell lines; D17, OSCA2, OSCA16, and OSCA50. Cells were treated with DHA at various concentrations for 48 hours and cell viability determined using the CyQuant™ assay. DHA decreased cell viability in all four canine osteosarcoma cell lines in a dose-dependent manner (Figure 3.1). A significant decrease in cell viability was observed at the concentrations of 5, 50, 10, and 10 μ M or greater for D17, OSCA2, OSCA16, and OSCA50 cell lines, respectively. The calculated IC₅₀ (95% CI) for the three cell lines were 8.7, 43.6, 16.8, and 14.8 μ M for D17, OSCA2, OSCA16, and OSCA50, respectively. Microscopic examination revealed cellular fragmentation and loss of attachment to the cell culture plate in all four cell lines (Figure 3.2). In contrast, DMSO vehicle-treated cells showed no evidence of cytotoxicity.

3.4.2. Induction of cell death through apoptosis

The mode of DHA-induced cell death was assessed using an ELISA based technique for the detection and quantification of cytoplasmic histone associated DNA fragments in the form of mononucleosomes or oligonucleosomes. Histone associated DNA fragments are generated during the process of apoptosis, and are thus represent a quantitative measure of this process. As demonstrated in Figure 3.3, DHA treatment induced a dose-dependent increase in free cytoplasmic nucleosome formation. A significant increase in cytoplasmic nucleosomes was observed at the concentration of 10 μ M in the D17, OSCA16, and OSCA50 cell lines, and at the concentration of 40 μ M in the OSCA2 cell line. This was accompanied by activation (cleavage) of caspase 3 (Figure 3.4). Caspase 3 is one of the effector caspases, which play key roles in cellular apoptotic pathway. Activation of caspase 3 is indicative of apoptosis. Taken together, these data indicate the anti-proliferative effect of DHA was, at least in part, due to apoptosis of treated cells.

3.4.3. Accumulation of subG₀ cells and G₂/M cells

We also investigated the effect of DHA on cell cycle progression. As shown in Figure 3.5, 3.6A-D, and Table 3.1, an increased proportion of subG₀/G₁ and G₂/M phase populations was observed in all four cell lines after DHA treatment, suggesting G₁/S phase specific cytotoxicity and induction of apoptosis (Figure 3.5, Figure 3.6A-D, Table 3.1).

3.4.4. Generation of reactive oxygen species by an iron-dependent mechanism

Finally, in order to determine if DHA induced iron-mediated ROS generation in canine osteosarcoma cells, we measured induction of ROS by DHA in the D17 canine osteosarcoma cell line (Figure 3.7A-B). 6-carboxy-2',7'-dichlorodihydrofluorescein is a cell-permeating non-fluorescent probe that, after oxidation by ROS, exhibits fluorescence which can be measured using flow cytometric analysis. Thus, in this assay an increase in fluorescence indicates an increase in the level of ROS. DHA induced a dose-dependent increase in ROS generation in D17 cells (Figure 3.7A). Significantly, this increase in ROS was suppressed by pretreatment of cells with the iron chelator desferrioxamine (Figure 3.7B), consistent with

previous studies regarding the role of iron in artemisinin cytotoxicity.

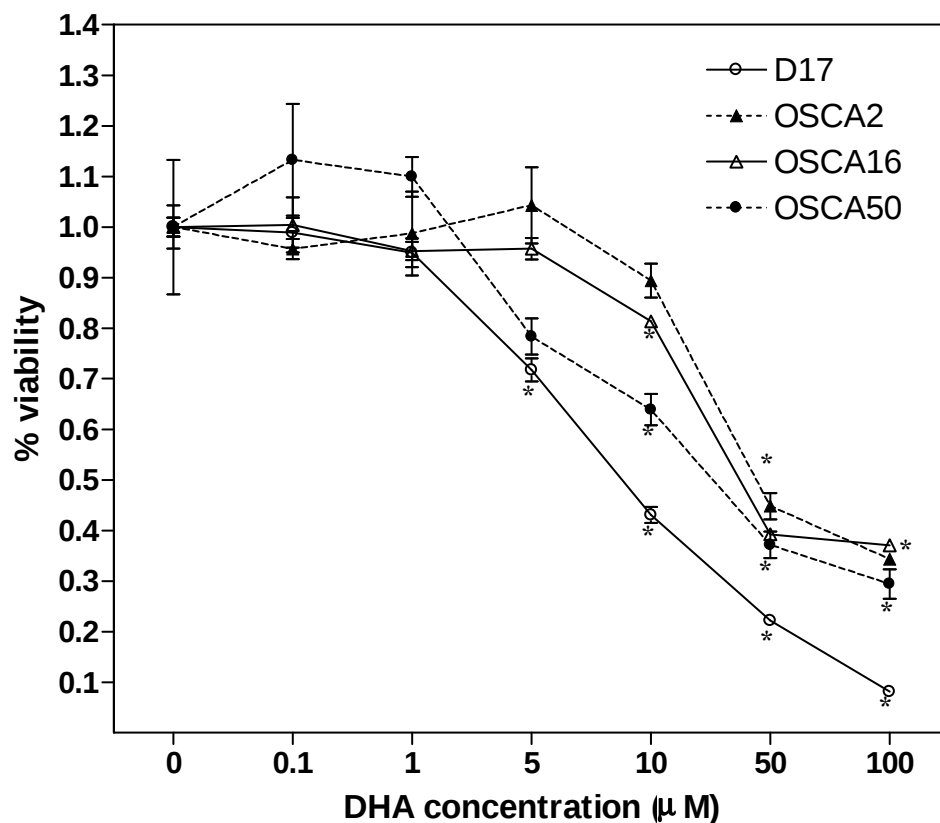


Figure 3.1. Percent viability curves of four canine osteosarcoma cell lines after 48 hours of exposure to DHA at varying concentrations. A significant ($p < 0.01$) decrease (*) in cell viability was seen at concentrations of $\geq 5 \mu\text{M}$ (D17), $10 \mu\text{M}$ (OSCA16, OSCA50), and $50 \mu\text{M}$ (OSCA2). Representative of three experiments.

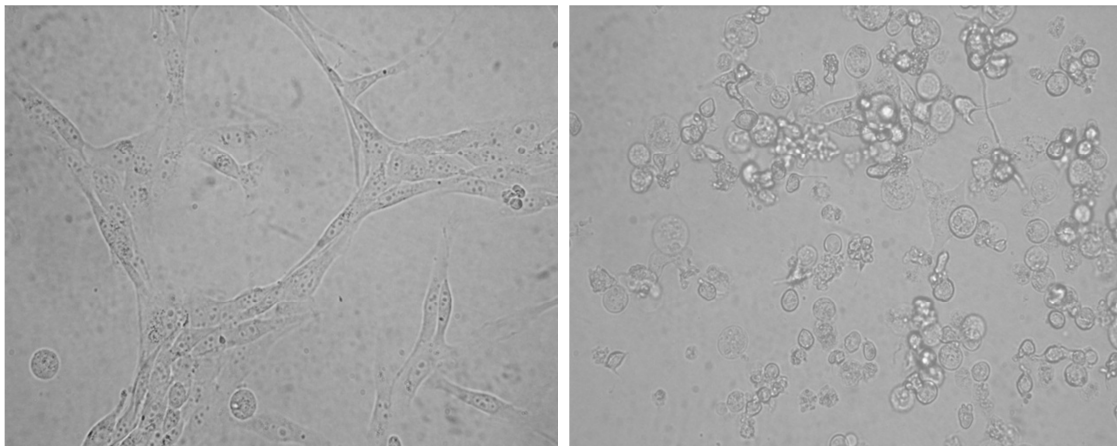


Figure 3.2. Morphologic changes in D17 cell lines after 48 hour exposure to 0.1% volume of DMSO (left) or DHA at a concentration of 10 μ M (right). Cellular fragmentation and detachment from the culture dish were seen with exposure to DHA.

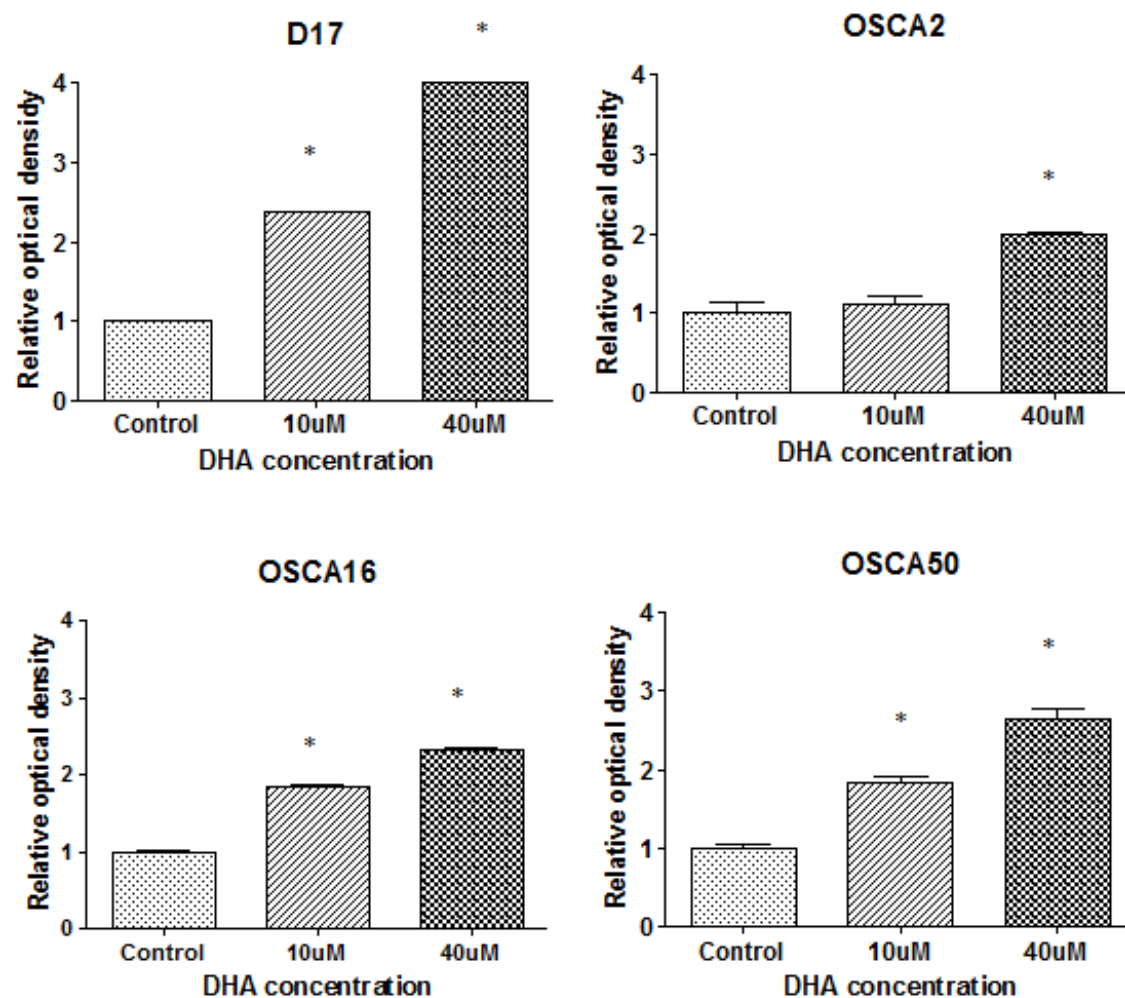


Figure 3.3. Formation of cytoplasmic nucleosomal DNA after 48 hour exposure to DHA. A significant ($p < 0.01$) increase (*) in cytoplasmic nucleosomes was seen at concentrations $\geq 10 \mu\text{M}$ in D17, OSCA16, and OSCA50 cell lines and at $40 \mu\text{M}$ in OSCA2 cell line. A repeated experiment showed similar result.

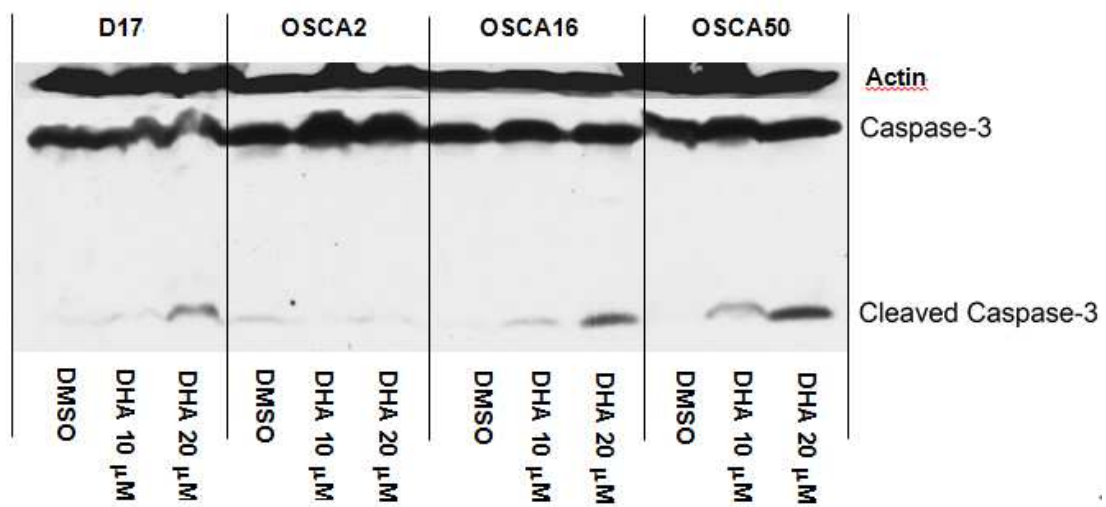


Figure 3.4. Western blot for caspase 3. Canine osteosarcoma cell lines were treated for 48 hours with DHA, protein lysates collected, and then immunoblotted with caspase 3 and β actin antibodies as described in Materials and Methods.

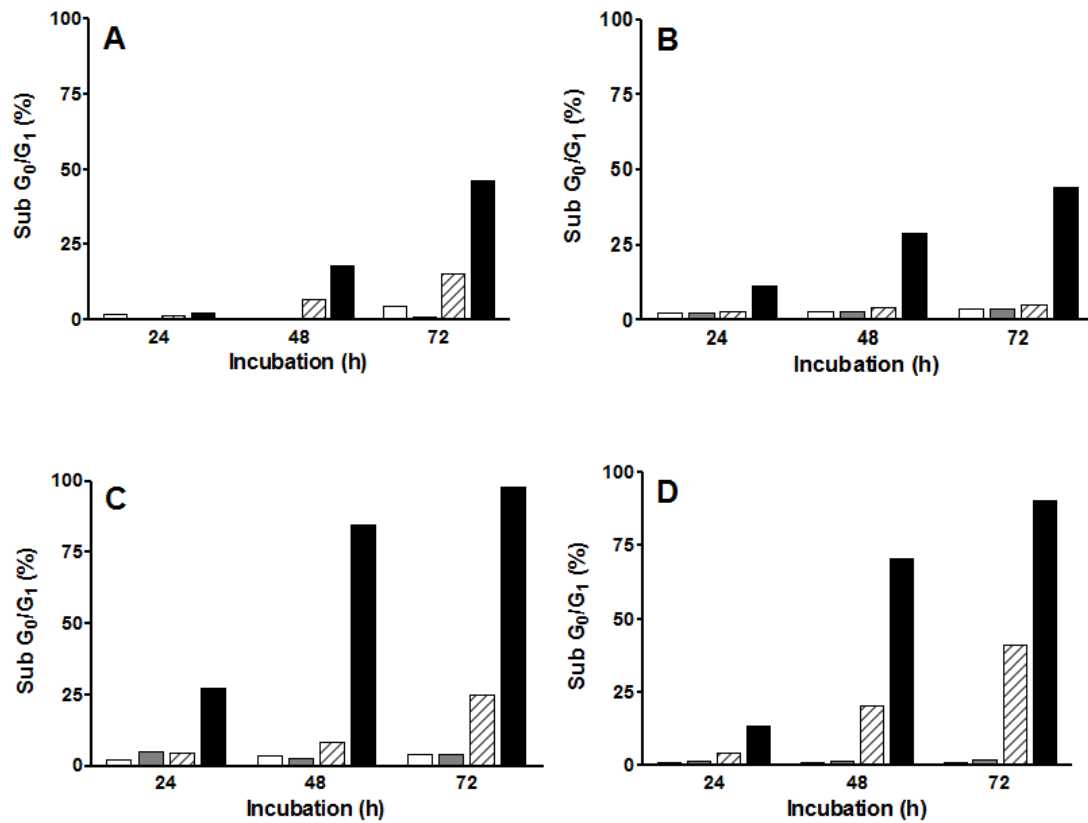


Figure 3.5. Proportion of subG₀/G₁ cells in canine osteosarcoma cell lines D17 (A), OSCA2 (B), OSCA16 (C), and OSCA50 (D) after incubation with DMSO (control treatment [white bar]) or various concentrations of dihydroartemisinin (1 μM [gray bar], 10 μM [diagonal-striped bar], 50 μM [black bar]) for 24, 48, or 72 hours. An increase in the subG₀/G₁ population was seen at concentrations of ≥ 10 μM in the D17, OSCA16, and OSCA50 cell lines, and at a concentration of 50 μM in the OSCA2 cell line. A repeated experiment yielded similar results.

Table 3.1. Cell cycle phase distribution after 48 hour exposure to 10 μ M DHA.

In all four cell lines, a significant ($p < 0.01$) decrease in the G_0/G_1 population and increase in the sub G_0/G_1 and G_2/M population were observed. A repeated experiment showed similar results.

Cell lines	Sub G_0/G_1	G_0/G_1	S	G_2/M
D17				
Control	1.3 %	46.2 %	13.0 %	39.5 %
DHA	10.2 %	16.8 %	12.3 %	60.7 %
OSCA2				
Control	2.3 %	50.1 %	10.3 %	37.3 %
DHA	3.9 %	40.6 %	10.1 %	45.4 %
OSCA16				
Control	2.3 %	39.7 %	9.3 %	48.7 %
DHA	7.6 %	29.5 %	7.2 %	55.7 %
OSCA50				
Control	0.9 %	55.1 %	21.8 %	22.2 %
DHA	6.8 %	24.9 %	22.3 %	46.0 %

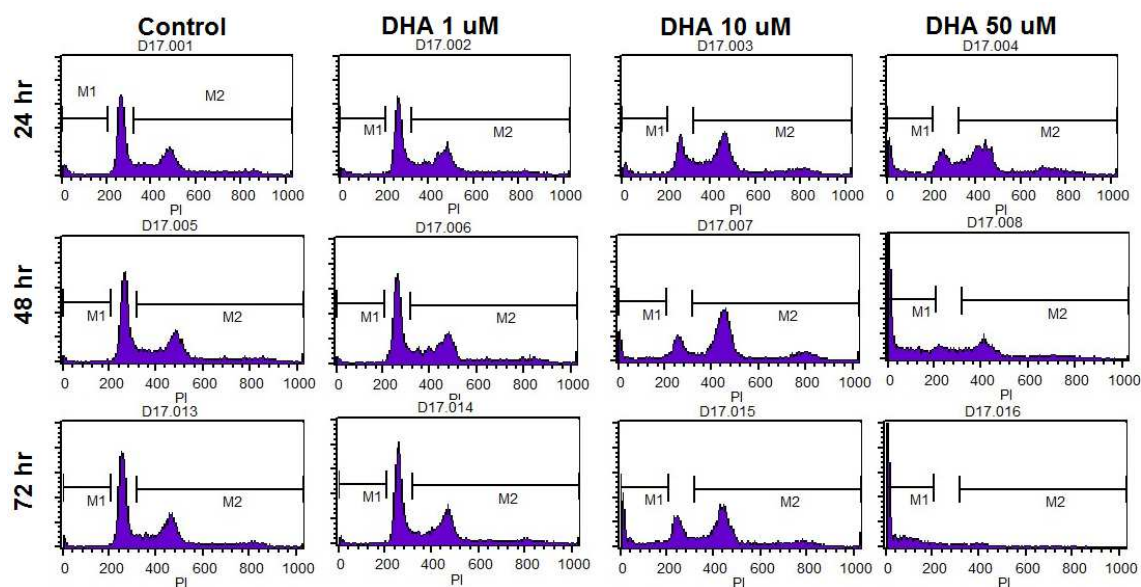


Figure 3.6A. Cell cycle phase distribution after 24, 48, and 72 hour exposure to 1, 10, or 50 μ M of DHA in D17 cells. A significant ($p < 0.01$) decrease in the G_0/G_1 population and increase in the sub G_0/G_1 and G_2/M population were observed. A repeated experiment showed similar results.

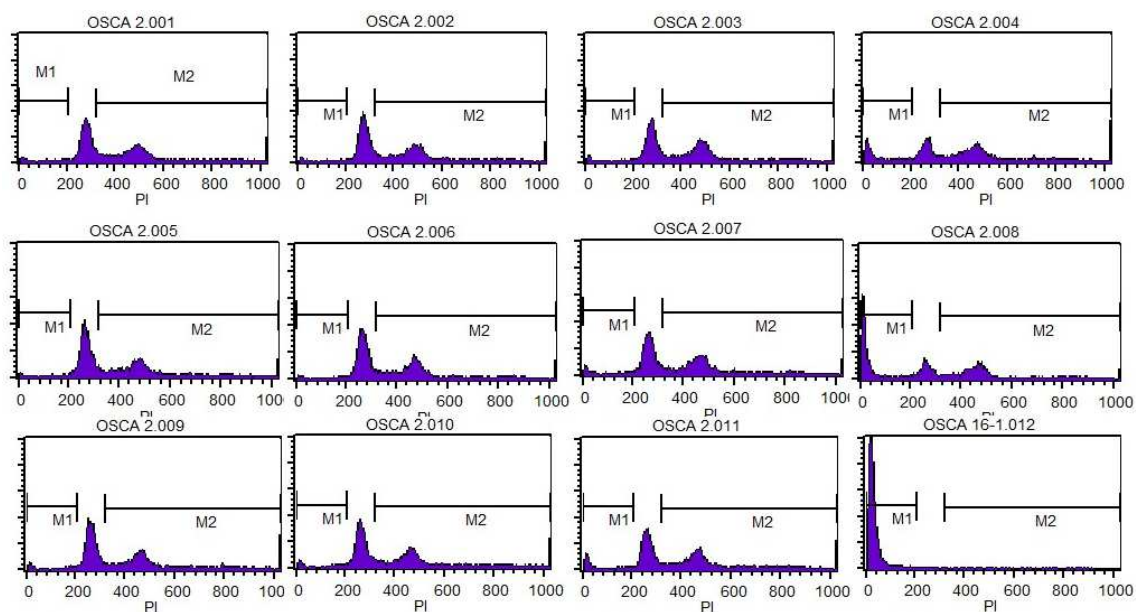


Figure 3.6B. Cell cycle phase distribution after 24, 48, and 72 hour exposure to 1, 10, or 50 μM of DHA in OSCA2 cells. A significant ($p < 0.01$) decrease in the G_0/G_1 population and increase in the sub G_0/G_1 and G_2/M population were observed. A repeated experiment showed similar results.

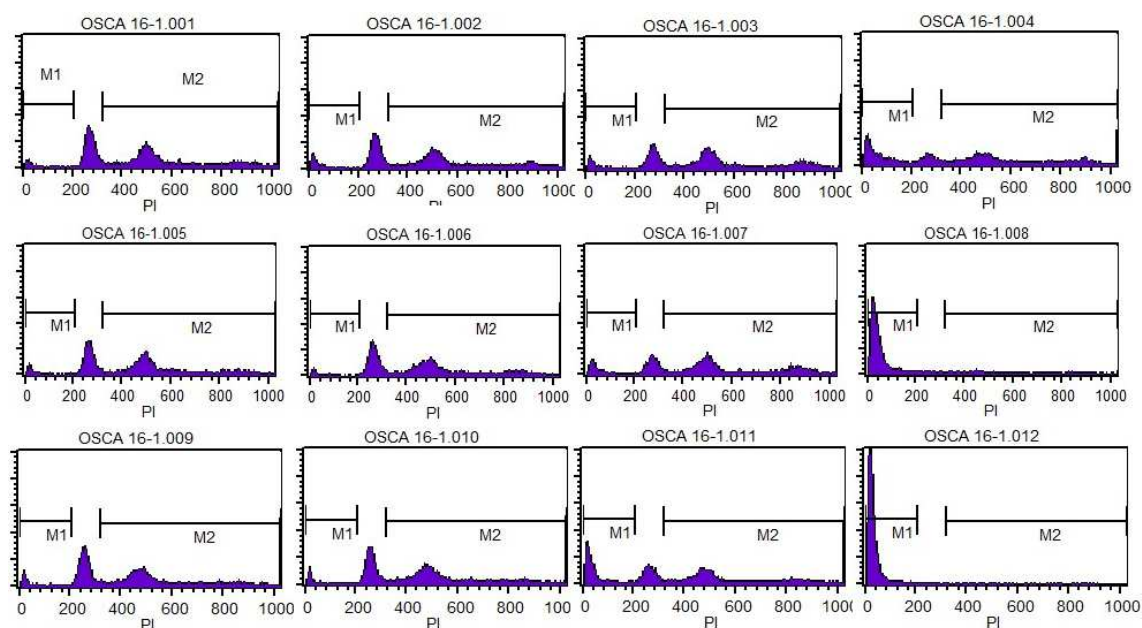


Figure 3.6C. Cell cycle phase distribution after 24, 48, and 72 hour exposure to 1, 10, or 50 μ M of DHA in OSCA16 cells. A significant ($p < 0.01$) decrease in the G_0/G_1 population and increase in the sub G_0/G_1 and G_2/M population were observed. A repeated experiment showed similar results.

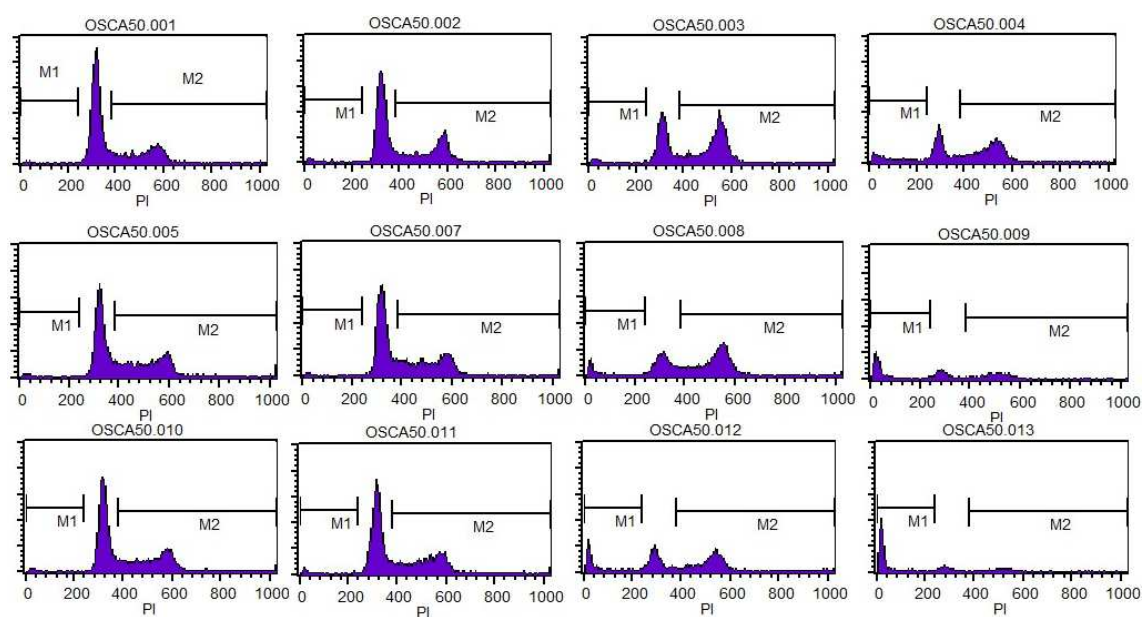


Figure 3.6D. Cell cycle phase distribution after 24, 48, and 72 hour exposure to 1, 10, or 50 μ M of DHA in OSCA50 cells. A significant ($p < 0.01$) decrease in the G_0/G_1 population and increase in the sub G_0/G_1 and G_2/M population were observed. A repeated experiment showed similar results.

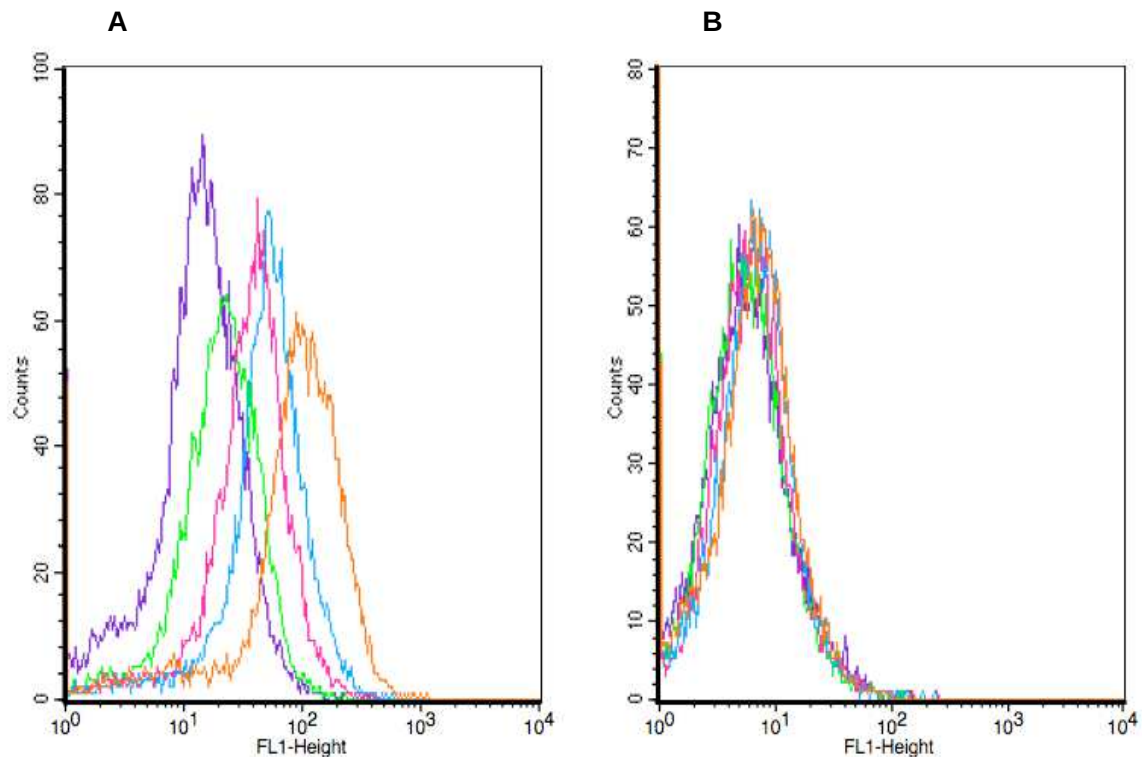


Figure 3.7. DHA induced iron-dependent formation of ROS. (A) D17 cells treated with 5 μM 6-carboxy-2',7'-dihydrofluorescein-diacetate (fluorescent probe for ROS) after a 12 hour exposure to DHA at a concentration of 0 (purple), 10 (green), 25 (red), 50 (blue), and 100 μM (orange). Cells were collected and analyzed by flow cytometry. A dose-dependent increase in production of ROS in response to DHA exposure is indicated by the right shift of the curves. (B) The same experiment with addition of the iron chelator desferroxamine to the culture medium. No shift of the curves was seen at all concentrations of DHA, indicating that the formation of ROS in response to DHA treatment is iron dependent.

3.5. Discussion

During the past two decades, the anti-malarial agent artemisinin and its derivatives have attracted interest as potential novel anticancer agents, cancer preventatives, multi-drug resistance reversal agents, and radiosensitizers. These artemisinin-derived 1,2,4-trioxanes exhibit significant activity in the nanomolar to micromolar range against a variety of human cancer cell lines. Their biologic activity in human cancer cell lines and apparently low toxicity in human malaria patients have stimulated interest in using artemisinin and its derivatives to treat canine cancers, particularly osteosarcoma, as the drug is readily available over-the-counter. However, to our knowledge, no study has evaluated the potential activity of artemisinin against canine cancer cells, either *in vitro* or *in vivo*.

In this study, a dose-dependent decrease in the viability of osteosarcoma cell lines was demonstrated after exposure to DHA. The IC₅₀ values for these osteosarcoma cell lines ranged from 8.7 to 43.6 μ M. These values are similar to those reported for three artemisinin derivatives (artesunate, artemether, and arteether) when screened with the National

Cancer Institute (NCI) 55 cell line panel (Efferth, 2002). In this panel, the greatest sensitivity was found in leukemia and colon cancer cell lines (mean IC_{50} : 1-2 μ M of artesunate) and the lowest sensitivity was found in non-small cell lung cancer cell lines (mean IC_{50} : 26 μ M of artesunate) (Efferth, 2002). Unfortunately, no sarcoma cell lines were tested.

Our study also demonstrated a dose-dependent increase in cytoplasmic nucleosomes and accumulation of activated caspase 3, consistent with DHA-induced apoptotic cell death. Furthermore, there was a dose-dependent increase in the sub G_0/G_1 cell population in treated cells, further supporting that the mechanism of DHA cell killing is, at least in part, due to apoptosis. Induction of apoptosis by artemisinin derivatives has been demonstrated in other studies evaluating human cancer cell lines (Efferth, 1996; Singh, 2004; Nam, 2007).

A decreased proportion of cells in the G_0/G_1 phase was observed in DHA treated cells. This is in accordance with previous reports, where the cytotoxicity of artesunate correlated with the proportion of the cells in G_0/G_1 phase (Efferth, 2003). The underlying mechanism for this cell cycle phase-selective cytotoxicity is currently unknown.

One of the postulated mechanisms of artemisinin and DHA-induced cytotoxicity is iron-mediated ROS generation, as is in its anti-malarial activity. Both antimalarial and tumoricidal activities of artemisinin are known to be iron-dependent (Disbrow, 2005; Meshnick, 2002). For example, the *in vitro* cytotoxicity of DHA in papilloma virus-infected epithelial cells could be reversed by chelating iron from the culture medium (Disbrow, 2005). To confirm that generation of ROS occurred in canine osteosarcoma cells, we measured ROS activity in the D17 canine osteosarcoma cell line treated with DHA. As expected, dose-dependent generation of ROS by DHA was observed. ROS generation was completely inhibited by the addition of desferroxamine, an iron chelating agent, to the culture medium, demonstrating that ROS generation by DHA is iron-dependent in canine osteosarcoma cells.

Our data indicate that DHA induced apoptosis of canine osteosarcoma cell lines requires drug concentrations in the micromolar range. Peak plasma concentrations after an antimalarial dose of oral DHA (4 mg/kg) in human malaria patients ranged from 1.9 to 16 μM (median: 4 μM) (Newton, 2002). These values are lower than the cytotoxic range observed in our study;

however, the low toxicity of artemisinins in human patients and the extremely high LD₅₀ values of artemisinin derivatives reported in a previous animal study suggests that cytotoxic plasma concentrations might be achievable *in vivo* by using higher doses of artemisinin derivatives than those currently being used empirically in dogs (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982). In summary, our data demonstrate biologic activity of DHA against canine osteosarcoma. Further studies to evaluate the safety and efficacy of artemisinin and its derivatives in dogs with osteosarcoma are warranted.

4. Cytotoxic Effects of Dihydroartemisinin in Canine Histiocytic Sarcoma Cell Lines: A Relationship Between Intracellular Ferrous Contents and Cytotoxicity of Dihydroartemisinin

4.1. Summary

Artemisinin-derived trioxanes has been shown to exhibit cytotoxic effect through iron-dependent generation of free radicals, thus making them potential candidates for cancer therapeutics for neoplasia with high iron-contents. Canine histiocytic sarcoma is a neoplasia of macrophage/dendritic origin. It retains hemophagocytic capability and causes erythrophagocytosis clinically called hemophagocytic syndrome, and therefore is suggested to have high iron content. In this study, we have evaluated cytotoxicity of dihydroartemisinin in two canine histiocytic sarcoma cell lines in relation to the cellular iron content. Dihydroartemisinin caused significant decrease in cell viability in both cell lines in a concentration-dependent manner. The mode of cell death was at least in part through induction of apoptosis. As predicted, dihydroartemisinin's

cytotoxicity was influenced by the cellular iron content; increasing intracellular iron content by adding ferrous citrate ammonium or holotransferrin resulted in enhanced cytotoxicity of dihydroartemisinin and decreasing cellular iron content by removing iron from the media by deferoxamine markedly obliterated the drug's effect. This study is the first to demonstrate *in vitro* cytotoxic activity of an artemisinin derivative in canine histiocytic sarcoma and suggests that neoplasm that contains high iron load may be appropriate candidate for artemisinin derivatives.

4.2. Introduction

Artemisinin-derived trioxanes has been shown to exhibit cytotoxic effect on various cancer cell lines through generation of free radicals and oxidation of intracellular targets such as transcriptionally controlled tumor protein (TCTP). Due to this unique mechanism of action, the cytotoxic effect of artemisinin-derivatives are generally more pronounced in neoplastic cells than their somatic counterparts because tumor cells overexpress transferrin receptor to increase uptake of iron to the cytoplasm and therefore contain higher iron contents than normal cells. However, some tumor cells are suspected to actively produce iron-binding protein such as ferritin and may contain increased amount of iron molecules independent of transferrin receptor expression. Hepatocellular carcinoma, lymphoma, and histiocytic sarcoma all have been known to cause hyperferritinemia; proposed underlying mechanism for this hyperferritinemia include erythrocyte destruction, alteration in erythropoiesis, release by tissue damage, injury to hepatocytes, and production by tumor cells.

Canine histiocytic sarcoma is a highly malignant tumor of phagocytic

macrophage or antigen-presenting dendritic origin (Affolter, 2002). The disease is rapidly progressive with limited success in therapeutic approach to intervene the course of disease (Skorupski, 2002; Rassnick, 2011). The reported response rates to 1-(2-chloroethyl)3-cyclohexyl-1-nitrosourea (CCNU), the current standard systemic chemotherapeutic agent, range from 29% to 46%, with median progression free interval of as short as 85-96 days (Skorupski, 2002; Rassnick, 2011). Without effective systemic treatment options, the overall prognosis of the affected dogs is guarded; even dogs with localized form of histiocytic sarcoma, the majority eventually develop disseminated metastatic lesions and succumb to disease within 6 months of diagnosis (Affolter, 2002; Craig, 2002; Fidel, 2006; Skorupski, 2009).

One unique aspect of canine histiocytic sarcoma is that the disease is known to cause marked hyperferritinemia supposedly due to erythrophagocytosis and production by the tumor cells; however, whether intracellular iron content is in fact elevated has not been elucidated. We hypothesized that canine histiocytic sarcoma cells, via direct erythrophagocytosis or ferritin production or both, contains high iron content than other histologic types of tumor, and the amount of intracellular iron

contents is directly associated with increased cytotoxicity of artemisinin-derivatives due to enhanced free radical generation. To test these hypotheses, we first quantified and semiquantified tissue iron contents by inductively coupled plasma-mass spectrometry (ICP-MS) and Berlin-blue stain, respectively. The relationship between intracellular iron concentration and cytotoxicity of dihydroartemisinin, cellular contents of iron molecules were manipulated and measured by ICP-MS, then anti-proliferative and apoptosis-inducing effects of dihydroartemisinin were compared between different conditions with either low, normal, and high cellular iron contents.

4.3. Materials and methods

4.3.1. Evaluation of tissue iron contents of canine histiocytic sarcoma

To test whether canine histiocytic sarcoma tissue contains higher amount of iron molecules compared to other tumor types, tumor tissue samples were collected from 7 dogs with histologically confirmed histiocytic sarcoma (Table 4.1) and 9 additional dogs with spontaneously occurring soft tissue neoplasia other than histiocytic sarcoma (Table 4.2).

For ICP-MS, 0.5 g of tumor tissue samples from each dog were washed by water and dried at 45 degrees for 12 hours. After adding 5 mL of 65% nitric acid^a and 1 mL of 30% hydrogenperoxide^b to the samples, tissue samples were decomposed using microwave sample decomposer^c. The processed samples were mess upped to the total of 10 mL by adding distilled water before used for ICP-MS. Amount of iron contents within each tumor sample was measured with ICP-MS^d. Briefly, the samples were ionized by collision with argon plasma, and amount of ferrous ion ($m/z = 56$) was detected and quantified within the sample.

Since ICP-MS measures the iron contents within the entire sample,

regardless of their origin (i.e., tumor cell or red blood cell, etc.), semiquantification of iron contents within the tumor cells was performed using Berlin-blue stain. Tissues were formalin-fixed and embedded in paraffin block and then tissue sections of 4 μm -thickness were prepared using rotary microtome^e. Deparaffinization of the tissue sections were routinely performed by dipping the samples in xylene^f for 5 minutes 3 times, then decreasing concentrations of ethanol solutions^g. Tissue sections were then used for Berlin-blue stain. A mixed solution of equal volumes of 2% potassium ferrocyanide^h and 0.7N hydrochloric acidⁱ was prepared immediately before each experiment. Appropriate amount of this solution was mounted on the tissue slides and incubated at room temperature for 60 minutes. Samples were washed with distilled water 5 times, and then counter-stained with 1% Safranin O^j in order to stain the nuclei.

4.3.2. Cell culture and cell lines

Two canine osteosarcoma cell lines (CHS-4 and DH82) were evaluated. The CHS-4 cell line (Azakami, 2006) was kindly provided by Dr. Makoto Bonkobara (Japan Veterinary and Life Science University). The

DH82 cell line was commercially purchased from European Collection of Cell Cultures, Salisbury, UK. CHS-4 cell line is derived from canine histiocytic sarcoma of dendritic origin, and DH82 is derived from canine histiocytic sarcoma of phagocytic macrophage origin. Both cell lines were cultured in Dulbecco's modified Eagle medium^k (DMEM) supplemented with 10% fetal bovine serum^l (FBS) and antibiotics (penicillin^m [100 U/ml] and streptomycinⁿ [0.1 mg/ml]) with the addition of 12 mM of 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)^o. Both cell lines were maintained in a humidified environment at 37° C under 5% CO₂ and 95% room air.

4.3.3. Chemicals

Dihydroartemisinin^p was dissolved in 100% dimethylsulfoxide (DMSO) at the concentration of 100 mM as a stock solution, protected from light, and kept at -80° C for long-term storage. The stock solution was further diluted with DMSO prior to use in tissue culture, resulting in a consistent DMSO concentration of 0.1% volume. Dihydroartemisinin solutions were newly prepared from the stock solution for each experiment.

Ferric ammonium citrate^p was dissolved in DMEM in 200 μ M concentration and stored at -80 °C until use.

Holotransferrin^r was prepared in DMEM at a concentration of 1 mg/mL, and stored at 4 °C until use.

Deferoxamine mesylate^s was dissolved in distilled water at a concentration of 100 mM and was stored at 4 °C until use.

4.3.4. Assessment of cell viability

Cell viability was assessed using tetrazolium assay also known as MTT assay (Rubinstein, 1990). Cells were seeded in 96-well plates at density of 1,000 - 2,000 cells per well in triplicates to a final volume of 50 μ L/well. After 24 hours, 50 μ L of medium containing DMSO (control) or different concentrations of dihydroartemisinin was added to each well to create final dihydroartemisinin concentrations of 1, 5, 10, 25, 50, 75, and 100 μ M and the cells were incubated for 24 additional hours. After treatment, 20 μ L of 5 mg/mL 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazoliumbromide^t (MTT) was added and the cells were further incubated at 37 °C, 5% CO₂ for 4 hours to allow for cells to form tetrazolium crystals. After incubation, the

medium were gently suctioned, and 50 μ L of dissolving solution was added to each well. Dissolving solution was prepared by mixing 20% sodium dodecyl sulfate^u (SDS) with equal volume of N,N-dimethylformamide^v with pH adjusted to 4.7 by adding 11.5N hydrochloric acid. Optical density at a wavelength of 570 nm (OD_{570}) of each well was measured by microplate reader^w. Cell proliferation was expressed as a percentage of the control wells: OD_{570} of sample/ OD_{570} of control (DMSO only) wells. Experiments were repeated at least three times.

4.3.5. Detection of apoptosis

Number of apoptotic cells were semiquantified by fluorescent staining using FITC-conjugated annexin V staining kit^x according to the manufacturer's instruction. Briefly, cells were suspended in binding buffer containing annexin V and propidium iodide and incubated for 5 minutes in a dark room. Cells were then fixed with 2% formaldehyde and blocked with 10 % rabbit serum. Cells were stained with fluorescein conjugate^y for 15 minutes for observation. Microscopic inspection of the cell membrane and nuclei was performed using a fluorescein microscopy^z.

4.3.6. Manipulation and measurement of intracellular iron contents

To increase the intracellular iron contents, 200 μ M of ferric ammonium citrate or 1 mg/mL of holotransferrin was added to the culture media. Cells were incubated for 24 hours (in case of ferric ammonium citrate) or for 6 hours (in case of holotransferrin) to allow for iron uptake. In each condition, the final concentration of DMSO was kept at <0.1% in all experiments.

To decrease intracellular iron contents, ferrous ion was chelated by the addition of 50 mM of deferoxamine mesylate to the culture media for 6 hours before each experiment. The final concentration of DMSO was kept at <0.1% in all experiments.

Measurement of intracellular iron contents were carried out using ICP-MS. Cells pretreated in above conditions were washed three times with isotonic phosphate buffer solution (pH 7.4). 5.0×10^5 cells were counted and cell pellet was created by centrifuging at 200xg for 5 minutes. After removing the supernatant, 800 μ L of 65% nitric acid and 200 μ L of 30% hydrogen peroxide were added and the samples were incubated at room temperature

for 24 hours. After incubation, 2% nitric acid was added to make the final volume of each sample to 8 mL for ICP-MS measurement. The rate of retrieval of each sample was confirmed by using a substance with known iron contents (DORM-3^{aa}).

4.3.7. Statistical analysis

Changes in cell viability, measured by tetrazolium assay, were compared using one-way analysis of variance. A pairwise multiple comparison procedure was performed using the Dunnet test. All statistical analysis was performed using GraphPad Prism 4.0 software^{ab}. Statistical significance was established as $P \leq 0.05$.

4.3.8. Footnotes

^a Nitric acid, Kanto Chemicals, Tokyo, Japan

^b Hydrogen peroxide, Kanto Chemicals, Tokyo, Japan

^c SpeedWaveTwo, BERGHOF, Eningen, Germany

^d ICP-MS Agilent 7700, Agilent Technologies, Santa Clara, CA

^e HM325, MICROM, Waltham, MA

- ^f Xylene, Wako Chemicals, Osaka, Japan
- ^g Ethanol, Wako Chemicals, Osaka, Japan
- ^h Potassium ferrocyanide solution, Muto Chemicals, Tokyo, Japan
- ⁱ Hydrochloric acid, Muto Chemicals, Tokyo, Japan
- ^j 1% Safranin O, Muto Chemicals, Tokyo, Japan
- ^k DMEM, GIBCO BRL, Gaithersburg, NY
- ^l FBS, Nichirei Bioscience, Tokyo, Japan
- ^m Penicillin, Nakarai Tesque, Kyoto, Japan
- ⁿ Streptomycin, Nakarai Tesque, Kyoto, Japan
- ^o HEPES, Chemical Dojin, Kumamoto, Japan
- ^p Dihydroartemisinin, Sigma-Aldrich Inc., Saint Louis, MO
- ^q Ferric ammonium citrate, Sigma-Aldrich Inc., Saint Louis, MO
- ^r Holtransferrin, Sigma-Aldrich Inc., Saint Louis, MO
- ^s Deferoxamine mesylate, Novartis Pharma, Tokyo, Japan
- ^t MTT, Wako Chemicals, Osaka, Japan
- ^u Sodium dodecyl sulfate, Wako Chemicals, Osaka, Japan
- ^v N,N-dimethyl formamide, Wako Chemicals, Osaka, Japan
- ^w Multiskan FC, Thermo Fisher Scientific, Waltham, MA

^x Annexin V-Biotin Apoptosis Detection Kit, BioVision Inc., Milpitas, CA

^y Fluorescein Conjugate, Birmingham, Southan Biotech, AL

^z FLoid™ cell imaging station, Life Technologies Japan, Tokyo, Japan

^{aa} DORM-3 Fish protein, Certified Reference Material, National Research
Council of Canada, Ottawa, Canada

^{ab} Graphpad Prism 4.0, GraphPad Software Inc., La Jolla, CA

Table 4.1. The origin of the tumor tissue samples from dogs with histologically confirmed histiocytic sarcoma. Samples were collected from 7 dogs diagnosed as histiocytic sarcoma at Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University between April 2011 and October 2012.

Dog	Age (yr)	Sex	Breed	Location
1	6	M	Golden Retriever	Pelvis
2	8	F	Flat-coated Retriever	Forelimb
3	8	M	Bernese Mountain Dog	Lungs
4	9	F	Welsh Corgi Penbroke	Lungs
5	10	M	Bernese Mountain Dog	Lungs
6	11	M	Golden Retriever	Diaphragm
7	5	F	Bernese Mountain Dog	Lungs

Table 4.2. The origin of the tumor tissue samples from dogs with histologically confirmed tumors other than histiocytic sarcoma. Samples were collected from 9 dogs diagnosed at Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University between April 2011 and October 2012.

Dog	Age (yr)	Sex	Breed	Histologic type	Location
8	Unknown	F	Miniature Duchshund	Adenocarcinoma	Lungs
9	9	F	Bull Terrier	Osteosarcoma	Hindlimb
10	11	M	Miniature Schnauzer	Hemangiosarcoma	Spleen
11	11	F	Shiba Inu	Fibrosarcoma	Thoracic wall
12	14	F	Mixed	Mast cell tumor	Forelimb
13	10	M	Mixed	Squamous cell carcinoma	Lungs
14	13	M	Miniature Duchshund	Hepatocellular carcinoma	Liver
15	Unknown	F	Golden Retriever	Lymphoma	Spleen
16	8	M	Mixed	Malignant melanoma	Gingiva

4.4. Results

4.4.1. Tissue iron contents in canine histiocytic sarcoma

To test whether canine histiocytic sarcoma and other neoplasm tissue actually contains high amount of iron contents, direct measurement of tissue iron contents by ICP-MS was performed. In this method, both protein-bound and free ionized form of iron contents were measured, as well as extratumoral iron contents (e.g., iron contents of hemoglobin within red blood cell in tumor vasculature). The rate of iron retrieval was estimated as 95.8 +/- 6.1%, suggesting a sufficient rate of retrieval for tissue iron content measurement. The median iron contents of histiocytic sarcoma tissues was 142 µg/g tissue (range: 40 - 700 µg/g tissue) and the median iron contents of other tumor tissues was 321 µg/g tissue (range: 36 – 1,580 µg/g tissue). There was no significant difference between the two groups (p=0.21, Figure 4.1). The two highest values were observed in samples from hemangiosarcoma (1,581 µg/g tissue) and hepatocellular carcinoma (1,195 µg/g tissue).

The results of Berlin-blue staining were summarized in Table 4. All histiocytic sarcoma tissues and majority of the other tumor tissue samples

contained Berlin-blue-positive cells.

4.4.2. Effects of intracellular iron concentration on cytotoxic activity of dihydroartemisinin in canine histiocytic sarcoma cells

Dihydroartemisinin showed significant cytotoxicity in both cell CHS-4 and DH82 cell lines in a concentration-dependent manner (Figure 4.2 and 4.3). The estimated IC₅₀ value for CHS-4 and DH82 cells were 10 μ M and 7.6 μ M, respectively.

To investigate whether cytotoxicity of dihydroartemisinin correlates with intracellular iron concentration, cells were incubated in regular DMEM, with or without addition of deferoxamine, an iron-chelator, ferric ammonium citrate, or holotransferrin. In each conditions, the amount of intracellular iron was quantified by ICP-MS. In both CHS-4 and DH82 cell lines, intracellular iron contents decreased with the addition of deferoxamine and increased with the addition of ferric ammonium citrate and holotransferrin (Figure 4.4 and 4.5). Although the difference did not reach statistical significance, there was a clear tendency for the cellular iron contents to decrease with pretreatment with deferoxamine and to increase with

preincubation with holotransferrin.

These increase or decrease in cellular iron concentration directly correlated with cytotoxicity of dihydroartemisinin. As shown in Figure 4.6 and 4.7, the sensitivity of both CHS-4 and DH82 cells to dihydroartemisinin were enhanced by increasing intracellular iron contents by ferric ammonium citrate, and the effect of dihydroartemisinin was obliterated by chelating iron from the media by deferoxamine. Similarly, the cytotoxic effect of dihydroartemisinin was enhanced by pretreatment with holotransferrin. These changes were more pronounced in DH82 cells, a cell line with stronger phagocytic capacity.

Dihydroartemisinin treatment also induced cellular apoptosis in a dose-dependent manner, demonstrated by increased number of annexin-positive cells as shown in Figure 4.10. Addition of ferric ammonium citrate and holotransferrin both decreased the required dihydroartemisinin concentration to induce significant apoptosis. Due to excessive cell death, few viable cells were observed at the concentration of 100 μ M in these conditions. In contrast, pretreatment with deferoxamine markedly inhibited the effect of dihydroartemisinin, resulting in no discernible apoptosis at 10 μ M, and

significant apoptosis was only seen at a concentration of 100 μ M.

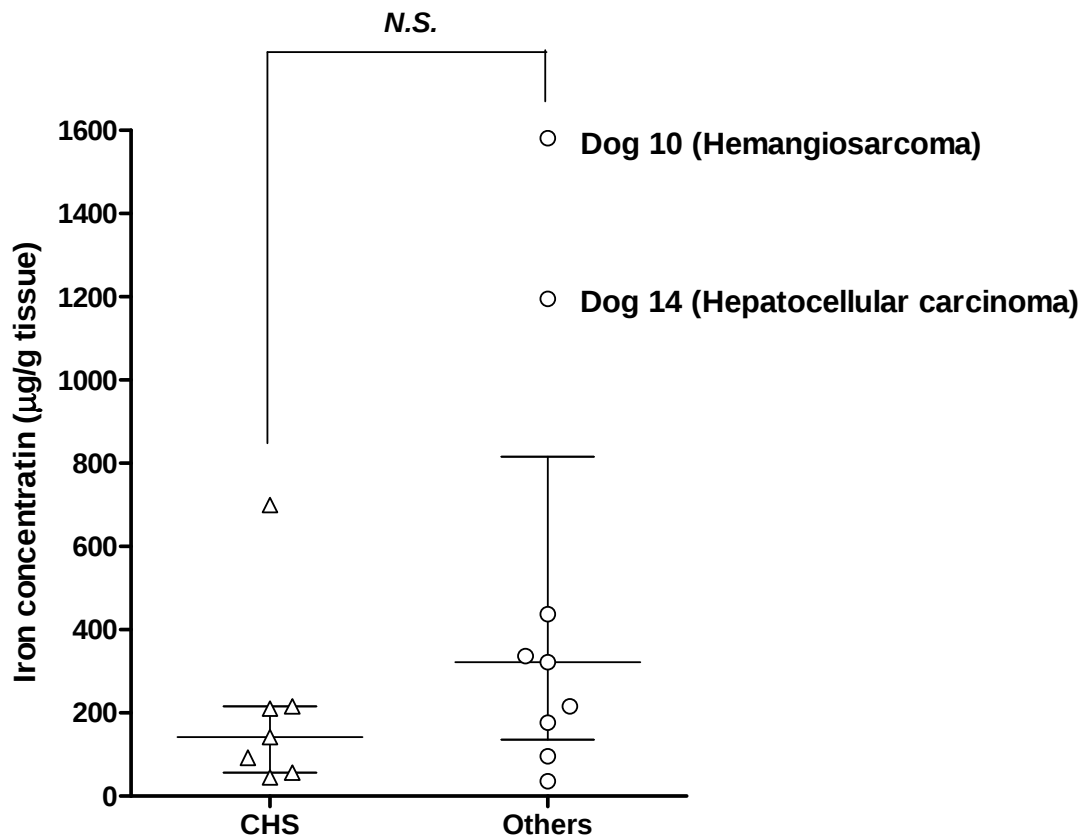


Figure 4.1. Tissue iron concentration of clinical samples from 7 canine histiocytic sarcoma tissues and 9 canine tumor tissues other than histiocytic sarcoma. There was no significant difference in tissue iron concentration between canine histiocytic sarcomas and other tumor types ($p=0.21$). Bars represent the median and interquartile values.

Table 4.3. Berlin-blue staining positivity of the canine tumor tissue samples.

All histiocytic sarcoma tissue samples and some of other tumor types contained Berlin-blue positive neoplastic cells although the majority of the neoplastic cells within the sample were stained negative

Dog	Histologic type	Presence of positive cells
1	Histiocytic sarcoma	+
2	Histiocytic sarcoma	+
3	Histiocytic sarcoma	+
4	Histiocytic sarcoma	+
5	Histiocytic sarcoma	+
6	Histiocytic sarcoma	+
7	Histiocytic sarcoma	+
8	Adenocarcinoma	+
9	Osteosarcoma	-
10	Hemangiosarcoma	+
11	Fibrosarcoma	-
12	Mast cell tumor	-
13	Squamous cell carcinoma	+
14	Hepatocellular carcinoma	+
15	Lymphoma	+
16	Malignant melanoma	-

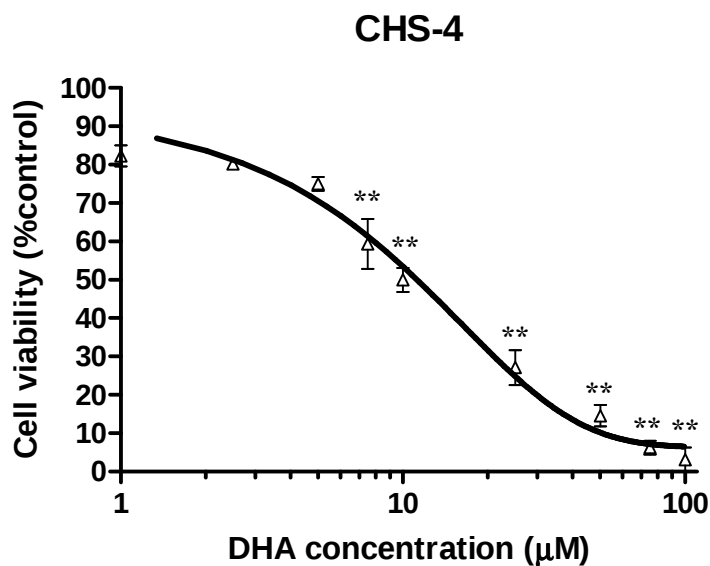


Figure 4.2. Changes of cell viability of CHS-4 cells with exposure to different concentrations of dihydroartemisinin. Cell viability decreased in a concentration-dependent manner with IC_{50} of 10 μM . Statistically significant decrease in cell viability was observed at concentrations above 7.5 μM . Repeated experiments showed similar results. DHA: dihydroartemisinin.

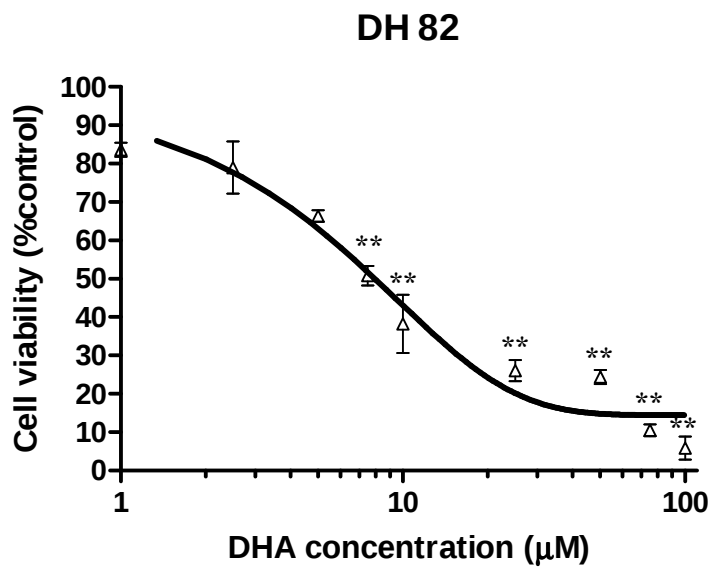


Figure 4.3. Changes of cell viability of DH82 cells with exposure to different concentrations of dihydroartemisinin. Cell viability decreased in a concentration-dependent manner with IC_{50} of 7.6 μ M. Statistically significant decrease in cell viability was observed at concentrations above 7.5 μ M. Repeated experiments showed similar results. DHA: dihydroartemisinin.

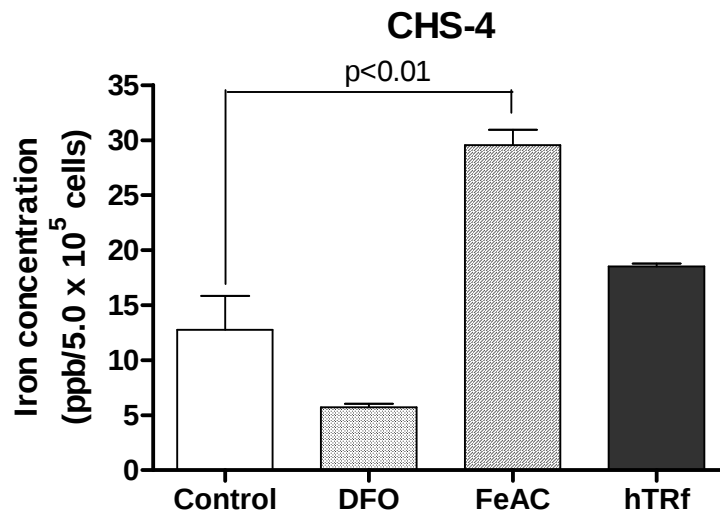


Figure 4.4. Intracellular iron concentration of CHS-4 cells. The intracellular iron contents was significantly increased when cells were preincubated with ferric ammonium citrate. The differences did not reach statistical significance in the other conditions; however, clear tendency was seen that treatment with deferoxamine decreases and holotransferrin increases cellular iron contents. DFO: deferoxamine, FeAC: ferric ammonium citrate, hTRf: holotransferrin.

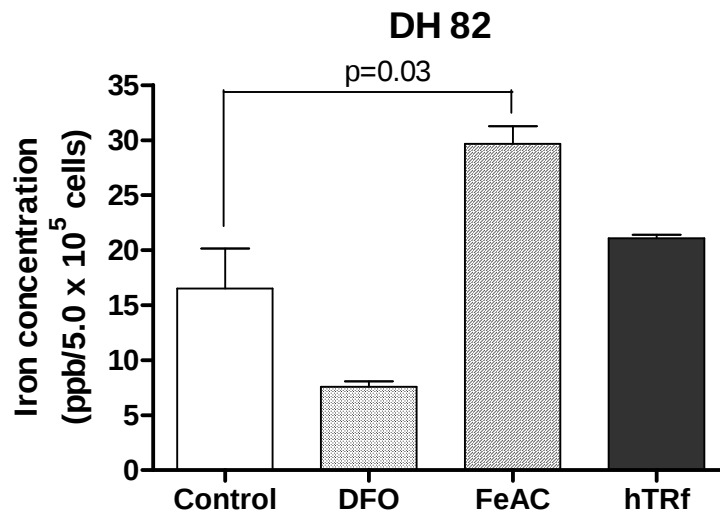


Figure 4.5. Intracellular iron concentration of DH82 cells. The intracellular iron contents was significantly increased when cells were preincubated with ferric ammonium citrate. The differences did not reach statistical significance in the other conditions; however, clear tendency was seen that treatment with deferoxamine decreases and holotransferrin increases cellular iron contents. DFO: deferoxamine, FeAC: ferric ammonium citrate, hTRf: holotransferrin.

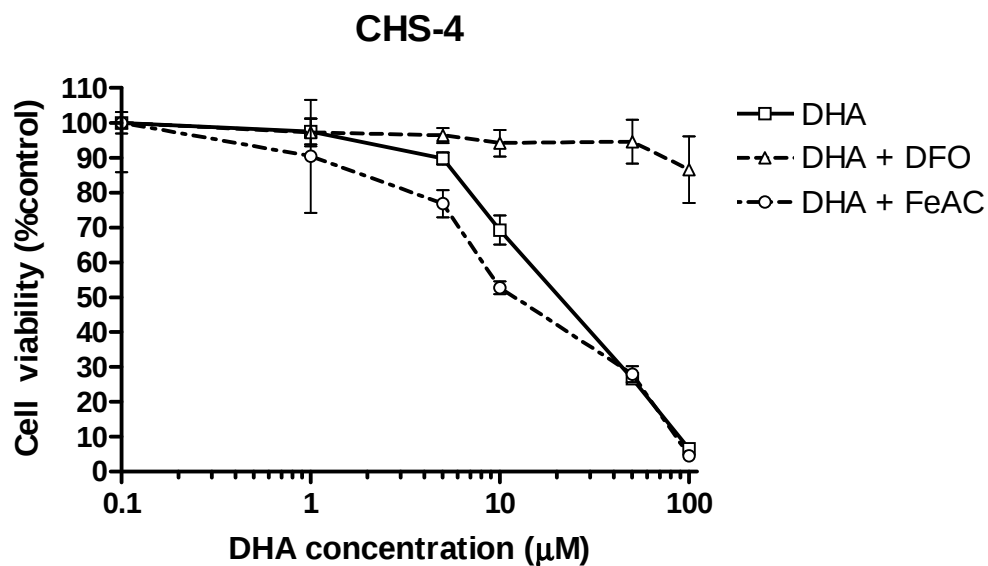


Figure 4.6. Cell viability of dihydroartemisinin-exposed CHS-4 cells cultured in normal DMEM (solid line), with addition of deferoxamine (dashed line), and with addition of ferric ammonium citrate (dotted line). The cytotoxicity of dihydroartemisinin was modestly enhanced by addition of ferric ammonium citrate. When iron was chelated by deferoxamine, the cytotoxicity of dihydroartemisinin was almost completely obliterated. DHA: dihydroartemisinin, DFO: deferoxamine, FeAC: ferric ammonium citrate.

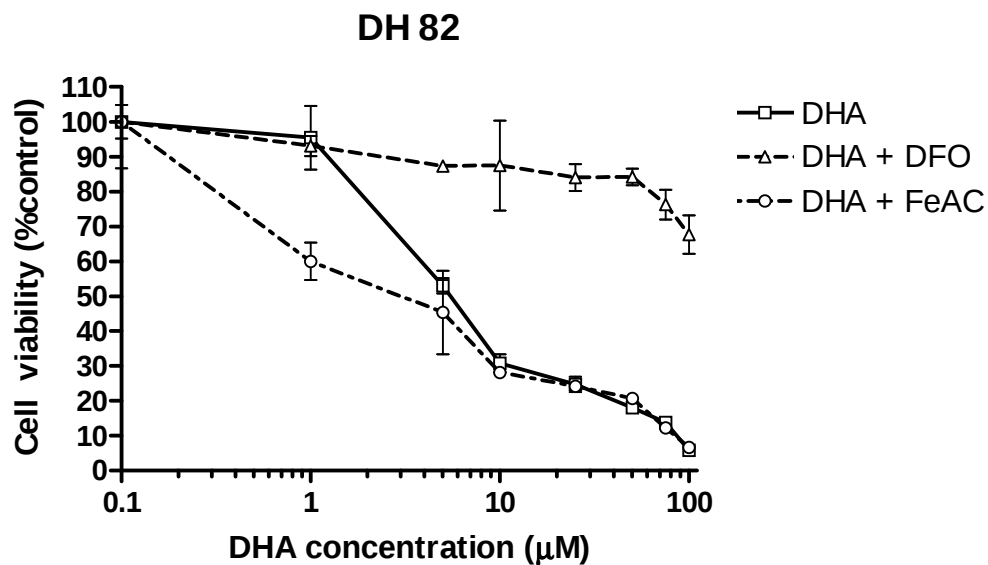


Figure 4.7. Cell viability of dihydroartemisinin-exposed DH82 cells cultured in normal DMEM (solid line), with addition of deferoxamine (dashed line), and with addition of ferric ammonium citrate (dotted line). The cytotoxicity of dihydroartemisinin was modestly enhanced by addition of ferric ammonium citrate. When iron was chelated by deferoxamine, the cytotoxicity of dihydroartemisinin was almost completely obliterated. DHA: dihydroartemisinin, DFO: deferoxamine, FeAC: ferric ammonium citrate.

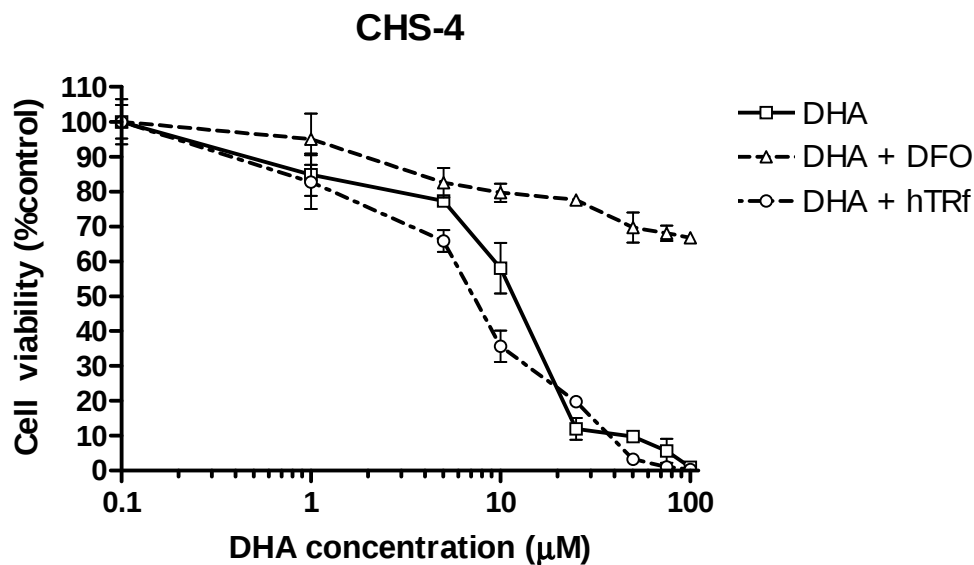


Figure 4.8. Cell viability of dihydroartemisinin-exposed CHS-4 cells cultured in normal DMEM (solid line), with addition of deferoxamine (dashed line), and with addition of holotransferrin (dotted line). The cytotoxicity of dihydroartemisinin was markedly enhanced by addition of holotransferrin. When iron was chelated by deferoxamine, the cytotoxicity of dihydroartemisinin was almost completely obliterated. DHA: dihydroartemisinin, DFO: deferoxamine, hTRf: holotransferrin.

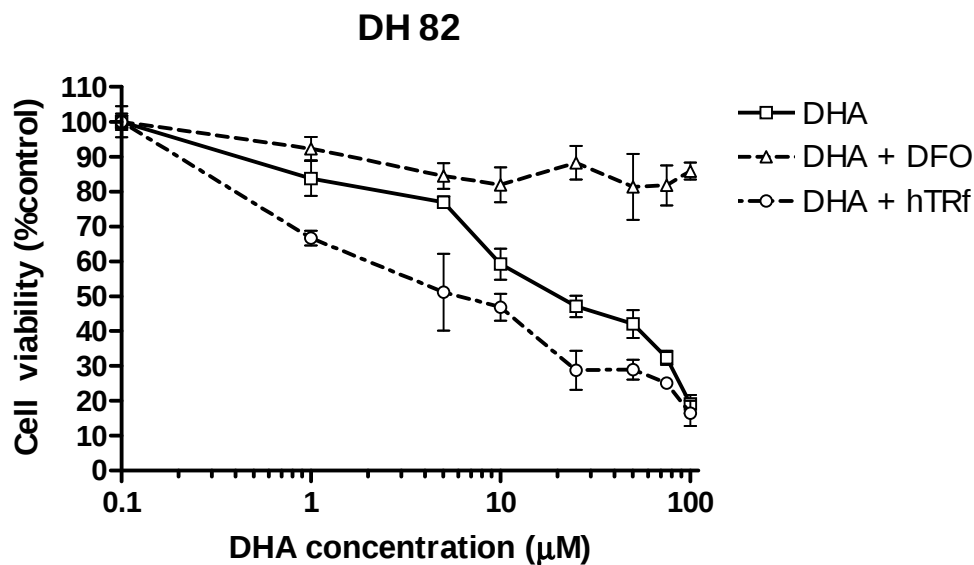


Figure 4.9. Cell viability of dihydroartemisinin-exposed DH82 cells cultured in normal DMEM (solid line), with addition of deferoxamine (dashed line), and with addition of holotransferrin (dotted line). The cytotoxicity of dihydroartemisinin was markedly enhanced by addition of holotransferrin. When iron was chelated by deferoxamine, the cytotoxicity of dihydroartemisinin was almost completely obliterated. DHA: dihydroartemisinin, DFO: deferoxamine, hTRf: holotransferrin.

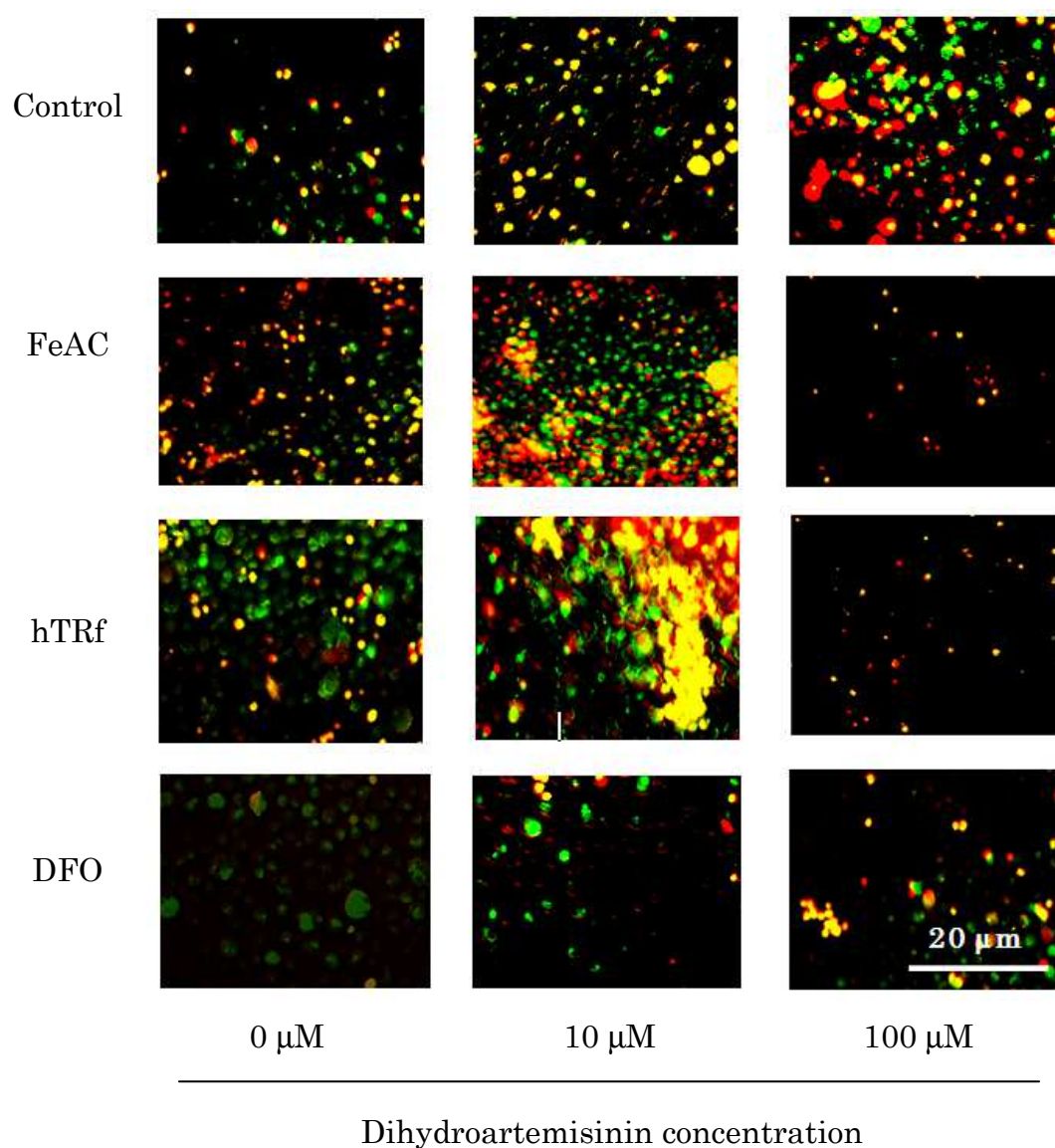


Figure 4.10. Fluorescent images of CHS-4 cells after 24 hours of exposure to 0, 10, and 100 μM of dihydroartemisinin. Green color represents FITC-labeled annexin V and red color represents propidium iodide. Yellow area represents co-localization of both annexin V and propidium iodide. Note that dihydroartemisinin induced cellular apoptosis all conditions except one with addition of diferoxamine. Similar result was seen in DH82 cells.

4.5. Discussion

Hyperferritinemia has long been recognized in several diseased conditions in humans including tumors of histiocytic origin (Yoda, 1980; Valberg, 1980; Esumi, 1989; Ya-You, 1998; Tang, 1987; Morita, 1981). Hyperferritinemia has also been investigated as an early diagnostic marker for canine histiocytic sarcoma (Friedrichs, 2010; Nielsen, 2011), and has been shown to exhibit marked hyperferritinemia similar to its human counterpart. However, whether this hyperferritinemia is caused by direct release from the tumor cells or through indirect mechanism such as erythrodestruction is unknown, and actual iron contents within the tumor tissue has not been elucidated. The result of this study indicated that canine tissue contents of iron in canine histiocytic sarcoma do not differ significantly from that of other tumor tissues. This is not surprising given the fact that all malignant tumor cells should contain increased intracellular iron contents through overexpression of transferrin receptors because iron is an essential cofactor for DNA synthesis. Furthermore, some of the evaluated tumor types are thought to have high iron contents; lymphoma in dogs is known to cause

hyperferritinemia and thus could have high tissue iron content (Friedrichs, 2010). Hepatocellular carcinoma, one of the two samples with extremely high iron content in this study, is a tumor of hepatocyte origin and thus expected to produce ferritin and have iron stores intracellularly. The other tumor sample with high iron contents was that of hemangiosarcoma. Unfortunately, ICP-MS could only evaluate the amount of iron in the entire sample and does not discriminate iron contents inside the tumor cells and hemoglobin-bound iron contents within red blood cells. Hemangiosarcoma tissue is primarily composed of abnormal neoplastic vascular space which is filled with high number of red blood cells or a large blood clot. Therefore, the reason for the high tissue iron concentration in our sample could either be due to high red blood cell contamination or due to true high iron contents within the tumor cells.

Histological evaluation of tissue sections with Berlin-blue staining can distinguish intracellular iron contents and other sources; however, this technique is qualitative rather than quantitative in nature, and the low sensitivity of this technique precluded detailed assessment of cellular iron contents. Many of the tumor samples, including all histiocytic sarcoma

samples evaluated, contained some Berlin-blue positive cells but majority of the tumor cells within the same section stained negative for Berlin-blue, indicating that not all tumor cells consistently have high intracellular iron load. One possible explanation is that Berlin-blue positive cells represent the tumor cells which have phagocytosed erythrocytes and those stained negative represents the tumor cells which have not. Therefore, the tissue iron concentration of histiocytic sarcoma not accompanied by clinical findings of hemophagocytic syndrome such as anemia, erythrodestruction, or thrombocytopenia, may have the similar level of tissue iron content as the other malignant neoplasia tissue. Unfortunately, none of the histiocytic sarcoma samples in this study were of phagocytic macrophage origin, and these dogs did not show clinical changes suggestive of hemophagocytosis. Tissue iron measurement and histological evaluation of intracellular iron contents of phagocytic histiocytic sarcoma samples are warranted to further clarify whether these tumors in fact have high intracellular iron contents through erythrophagocytosis.

The *in vitro* assay on canine histiocytic sarcoma cell lines of both dendritic cell origin and macrophage origin showed clear sensitivity of these

two cell lines to cytotoxic effect of dihydroartemisinin. The calculated IC₅₀ values were in low micromolar range, which is relatively low compared to other reported canine and human tumor cell lines (Efferth, 2002; Hosoya, 2008).

This cytotoxic effect of dihydroartemisinin on canine histiocytic sarcoma cell lines were further enhanced by supplementation of ferrous iron to the culture media in a form of ferric ammonium citrate, and abolished by chelating ferrous iron within the media by pretreating the cells with deferoxamine mesylate, thus providing a proof of concept that dihydroartemisinin exhibits cytotoxicity through iron-mediated mechanism. Experiments in the chapter 3 showed that dihydroartemisinin generates free-radicals through a mechanism mediated by iron; together, these findings indicate that dihydroartemisinin damage the cells through iron-mediated generation of free radicals and amount of intracellular ferrous iron contents is an important factor that determines the cellular sensitivity to cytotoxicity of dihydroartemisinin. Similar effect was seen when iron was supplemented by a form of holotransferrin, a form more closely resembles in vivo cellular environment. These effects were more pronounced in DH82 cells, a cell line

of phagocytic macrophage origin, and both treatment with ferric ammonium citrate and holotransferrin resulted in significant decrease in cell viability at the concentration as low as 1 μ M of dihydroartemisinin. Targeting tumors with naturally high iron content, artificially increasing intracellular iron contents, or exploiting cellular mechanism to uptake ferrous iron to increase the drug uptake, such as holotransferrin-conjugated dihydroartemisinin (Lai, 2005), all seem viable strategies to enhance the cytotoxicity of dihydroartemisinin for clinical use.

These decreases in cell viability were, at least in part, due to induction of apoptosis. In a normal culture condition, dihydroartemisinin caused slight increase in a number of cells undergoing apoptosis at 10 μ M and majority of the cells were undergoing apoptosis at 100 μ M. Increasing cellular iron contents with either ferric ammonium citrate or holotransferrin also enhanced this effect, resulting in marked induction of apoptosis at 10 μ M in both conditions. Increasing the concentration of dihydroartemisinin to 100 μ M resulted in near complete cell death and apoptosis could not be observed microscopically. As predicted, pretreatment with deferoxamine mesylate inhibited this induction of apoptosis, presumably via prevention of

iron-mediated free radical generation, again supporting our hypothesis.

These enhancement or inhibition of cytotoxicity of dihydroartemisinin correlated well with actual measured intracellular iron contents. In both cell lines, deferoxamine decreased and ferric ammonium citrate and holotransferrin increased cellular iron content, although the difference between the control did not reach statistical significance in deferoxamine and holotransferrin, suspectedly due to small sample size precluding detection of true difference (type II error). Despite the fact that pretreatment with holotransferrin enhanced cytotoxicity to a similar degree as treatment with ferric ammonium citrate, measured cellular iron content of holotransferrin-treated cells were somewhat lower than that of ferric ammonium citrate-treated cells in both cell lines. This is probably attributable to shorter preincubation time with holotransferrin. Cells were exposed only for 6 hours before intracellular iron measurement, whereas in cell viability and apoptotic assays, cells were preincubated for 6 hours and continued to be exposed to holotransferrin for additional 24 hours. To the author's knowledge, this study is the first to actually quantify the changes in intracellular iron concentration with iron supplementation or chelating in

relationship with enhancement or inhibition of cytotoxicity of dihydroartemisinin.

In conclusion, our study showed that tissues of many canine tumors including histiocytic sarcoma contains high iron contents, and thus could be candidates for dihydroartemisinin treatment, although the tissue iron content of non-phagocytic histiocytic sarcoma did not differ significantly from other tumor types. Furthermore, both phagocytic and non-phagocytic canine histiocytic sarcoma cell lines were shown to be susceptible to dihydroartemisinin with marked enhancement of cytotoxicity by increasing intracellular iron content. These results suggest that dihydroartemisinin can be a viable antineoplastic agent for treatment of canine histiocytic sarcoma. Further investigation toward its clinical use is warranted.

5. General Conclusion

These studies demonstrated clinical safety and antitumor activity of oral artemisinin in spontaneously occurring tumors in dogs. This is the first study to document the antineoplastic activity in a large animal model. However, the pharmacokinetic analysis indicated low bioavailability and incomplete conversion of orally administered artemisinin to active metabolite, dihydroartemisinin, suggesting that use of more potent derivatives such as artesunate and dihydroartemisinin and parenteral route of administration should be considered for future studies.

Antiproliferative and proapoptotic activities of dihydroartemisinin were also confirmed *in vitro* using four canine osteosarcoma cell lines and two canine histiocytic sarcoma cell lines. Estimated IC₅₀ values of these cell lines suggest the use of oral artemisinin with the dose that currently being used or higher is unlikely to achieve meaningful plasma concentration; however, by using more potent artemisinin-derivatives such as dihydroartemisinin and parenteral route of administration, plasma concentrations with antitumor activity may be achievable especially in tumor types with higher sensitivity such as canine histiocytic sarcoma.

An iron-mediated generation of free radicals, a proposed mechanism for artemisinin to exhibit cytotoxic activity against cancer cells, was successfully observed in a canine osteosarcoma cell line in a concentration-dependent manner, and this generation of free radicals could be completely prevented by chelating ferrous iron from the media, providing a proof of concept for this mechanism. Increase and decrease of intracellular ferrous iron content were associated with increased and decreased sensitivity to cytotoxic effect of dihydroartemisinin in canine histiocytic sarcoma cell lines, further confirming that cell damage by generation of reactive oxygen species was the mechanism of cytotoxicity and this required presence of ferrous iron within the cells.

Measurement of intratumoral iron contents showed that various tumor tissues in dogs including histiocytic sarcomas and hepatocellular carcinoma contains high amount of iron within the tissue, and can be candidates for the treatment with dihydroartemisinin. These findings lay basis for further research of artemisinin-derived trioxanes in treatment of malignant tumors in dogs.

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7. Japanese Summary

アルテミシニンとは、ヨモギ類植物である青蒿（セイコウ：*Artemisia annua*）より分離される薬効成分であり、アルテミシニン誘導体トリオキサンは、人マラリアに対する第一選択薬とされている。近年、アルテミシニンやその誘導体のヒト由来腫瘍細胞株に対する細胞障害作用が報告され、抗腫瘍薬としての研究が盛んに行われているが、犬由来腫瘍での報告はない。

本研究では、経口投与アルテミシニンの腫瘍罹患犬に対する安全性の検証および臨床的抗腫瘍効果ならびに薬物代謝の評価を行い、高い安全性と一部の自然発生腫瘍に対する腫瘍縮小効果を確認した。しかしながら、経口投与されたアルテミシニンの Bioavailability は低く、より活性の高いジヒドロアルテミシニンへの代謝は不完全にしか認められなかったため、他のアルテミシニン誘導体の非経口投与が望ましいと思われた。

ジヒドロアルテミシニンの *in vitro* での抗増殖作用およびアポトーシス誘導作用を 4 つの犬骨肉腫細胞株および 2 つの犬組織球性肉腫細胞株で確認した。得られた IC₅₀ 値から、抗腫瘍活性を有するジヒドロアルテミシニンの血中濃度は臨床的に達成可能であると思われたが、現在用いられているアルテミシニン薬用量よりも高い用量が必要であることが示唆された。

また、ジヒドロアルテミシニンの抗腫瘍効果発現機序と考えられている活性酸素による細胞障害について、犬骨肉腫細胞株を用いて実際に細胞内活性酸素量がジヒドロアルテミシニンの濃度依存性に上昇すること、さらに、この反応が培地内の鉄分をキレートすることで抑制されることを確認した。また、複数の犬組織球性肉腫細胞株を用いた実験にて、細胞内鉄量を増加または減少させることにより、ジヒドロアルテミシニンの細胞障害性に対する感受性が細胞内鉄量に比例して増加または減少することが確認され、上記実験結果と合わせて、ジヒドロアルテミシニンによる細胞障害作用は、鉄を介したフリーラジカルの産生およびそれによる細胞構造の損傷によると考えられた。

また、犬臨床例からの腫瘍サンプルを用いた組織内鉄含有量の測定では、組織球性肉腫および肝細胞癌組織を含む、複数の腫瘍組織サンプルにて高い鉄含有量が示された。本研究の結果は今後の犬腫瘍におけるアルテミシニン誘導体の抗腫瘍活性および臨床応用に関する研究の基礎となるものと考えられる。

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