Artemisinin is an antimalarial agent that gained prominence when its antimalarial properties were discovered in 1972. It is derived from the Chinese plant *Artemisia annua* and is used in traditional Chinese medicine for patients with chills and fever. Since then, it has been extensively studied as an antimalarial agent. Similar to malarial agents, artemisinins are believed to react with intracellular iron in free heme molecules within malaria-infected red blood cells (RBCs) and are converted to highly reactive free radicals. This reaction is attributed to generation of reactive oxygen species (ROS), which are believed to be relevant to the treatment of dogs with cancer.

Artquinghao, a decoction of *A. annua* with quinone compounds, has been used in traditional Chinese medicine for patients with chills and fever. Since 1972, when its antimalarial properties were discovered, artemisinin and its semisynthetic derivatives (ie, arteether, artemether, artesunate, artelinate, and artemiol [dihydroartemisinin]) have been studied extensively as antimalarial agents. These compounds react with intracellular iron in free heme molecules within malaria-infected RBCs and are converted to highly reactive free radicals. Their antimalarial activity is attributable to generation of ROS, which are believed to be alkylate 1 or more essential malarial proteins.

Artemisinins have antineoplastic properties. Similar to the antimalarial effects, the tumoricidal activity of artemisinins for neoplastic cells is secondary to generation of ROS. Because 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. This may explain the basis of the selective cytotoxic effects of artemisinins for neoplastic cells. For example, human leukemia and breast cancer cells are up to 100-fold more sensitive to the cytotoxic effects of artemisin than are normal human lymphocytes and breast epithelial cells. Several additional studies have revealed that artemisinin and its derivatives have cytotoxic effects against multiple human cell lines in vitro and against a rat fibrosarcoma cell line in vivo. Finally, the potential clinical efficacy of artemisinin derivatives has been indicated in case reports of humans with laryngeal squamous cell carcinoma, metastatic uveal melanoma, and pituitary macroadenoma. Together, these data suggest that artemisinin may be a useful agent to treat humans with cancer and could also be relevant to the treatment of dogs with cancer.
Given the low toxicity for artemisinin and the antineoplastic activity of artemisinin in humans with cancer and human cell lines, as well as anecdotal reports of efficacy in dogs with cancer (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, the study reported here was initiated to evaluate the in vitro effects of dihydroartemisinin, the active metabolite of most artemisinin derivatives, on cell viability, cytotoxicosis, and cell cycle progression in canine osteosarcoma cell lines.

**Materials and Methods**

**Sample population**—Four canine osteosarcoma cell lines (D17, OSCA2, OSCA16, and OSCA50) were evaluated. The D17 cell line was established from an osteosarcoma that had metastasized to the lungs of an 11-year-old female Standard Poodle. The OSCA cell lines were established from primary osteoblastic osteosarcomas (OSCA-2 was from a 2-year-old male Rottweiler, OSCA-10 was from a 10-year-old Rottweiler, and OSCA-50 was from a 7.5-year-old Saint Bernard).

**Cell culture**—Cells of the D17 cell line were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and antimicrobials (penicillin [100 U/mL] and streptomycin [0.1 mg/mL]). The OSCA cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and antimicrobials (penicillin [100 U/mL] and streptomycin [0.1 mg/mL]). All cell lines were maintained in a humidified environment of 95% room air and 5% carbon dioxide at 37°C. The doubling times of these cell lines for these culture conditions were approximately 24 hours for D17, OSCA-2, and OSCA-50 and 36 hours for OSCA-16.

**Dihydroartemisinin**—Dihydroartemisinin was dissolved in 100% DMSO at a concentration of 100mM to create a stock solution, which was then protected from light and kept in long-term storage at −80°C. The stock solution was further diluted with DMSO prior to use in tissue culture, which resulted in a consistent DMSO concentration of 0.1% per condition. Dihydroartemisinin solutions were newly prepared from the stock solution for each experiment.

**Assessment of cell viability**—Cells were seeded in 96-well plates at densities of 1,000 to 2,000 cells/well (final volume, 100 μL/well) in 6 replicates. After 24 hours, 100 μL of medium containing DMSO (control wells) or various concentrations of dihydroartemisinin was added to each well to create final dihydroartemisinin concentrations of 0.1, 1, 5, 10, 50, and 100μM, and the cells were incubated for an additional 48 hours. After that incubation, medium was removed by gentle suction, and plates were stored at −80°C until use. Cell proliferation was assessed by use of a nucleic acid fluorescence assay kit, which was conducted in accordance with the manufacturer’s instructions. After subtraction of background values, intensity of fluorescence of each reaction product was measured by use of a plate reader with excitation at 485 nm and emission detection at 530 nm. Cell proliferation was expressed as a percentage of the control wells by use of the following equation: (fluorescence of sample wells/fluorescence of control wells) × 100. Experiments were repeated a minimum of 3 times.

**Assessment of nucleosome fragmentation (cell death)**—To assess drug-induced apoptotic cell death, an ELISA kit was used in accordance with the manufacturer’s instructions. The ELISA was 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 to 5.0 × 10⁴ osteosarcoma cells were cultured in medium in 6-well plates for 24 hours before treatment. Cells were incubated with various concentrations of dihydroartemisinin (0 to 40μM) or DMSO (control wells) for 24 hours. Then cells were collected, and 1.0 × 10⁴ cells were counted and used in the ELISA.

**Western immunoblot analysis**—Osteosarcoma cell lines were incubated with various concentrations of dihydroartemisinin or DMSO (control wells) and collected after 24 hours of exposure. Cells were lysed in protein extraction reagent, unless otherwise stated. Additionally, a protease inhibitor cocktail mixture and phosphatase inhibitors were added to the lysis buffer. Cells were washed with ice-cold PBS solution and resuspended in lysis buffer containing 20mM Tris-HCl (pH, 8.0), 137mM NaCl, 1mM CaCl₂, 10% glycerol, 1% nonionic detergent, 0.5% deoxycholate, 0.1% SDS, 100μM (2-aminoethyl)-benzenesulfonylfluoride, leupeptin at 10 μg/mL, and aprotinin at 10 μg/mL. After protein quantitation, equivalent amounts of proteins (50 to 100 μg) from each lysate were resolved on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were washed with TBST, blocked by incubation in 5% skim milk in TBST for 1 hour at 20°C, and probed with antibodies specific for caspase 3 or β-actin (both at a dilution of 1:500 in TBST and 5% bovine serum albumin) by incubation overnight at 4°C. Membranes were then washed and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (dilution of 1:15,000) in 2.5% milk in TBST for 1 hour. Blots were then developed by use of a labeling reagent.

**Cell-cycle analysis**—For detection of cell-cycle progression and cell death, cell lines were seeded in 6-well plates at a density of 2.0 × 10⁴ cells/well. Cells were incubated for 24 hours, after which the medium was replaced with medium containing DMSO (control wells) or 1, 10, or 50μM dihydroartemisinin. Plates were then incubated for 24, 48, or 72 hours, after which cells were collected, washed with PBS solution, and fixed in 70% ethanol. Cell suspensions were stored overnight at 4°C. Fixed cells were washed in 5 mL of PBS solution and suspended in 500 μL of PBS solution containing RNase A (100 μg/mL) and propidium iodide (50 μg/mL). Flow cytometric analysis was performed. Data were analyzed by use of commercially available software. Accumulation of cells in the sub G₁/G₀ phase of the cell
cycle, an indicator of DNA fragmentation and apoptosis, was used to quantify cell death. Experiments were repeated 2 times.

**Measurement of ROS**—Production of ROS was determined by use of 6-carboxy-2',7'-dichlorodihydrofluorescein and flow cytometric analysis, as described elsewhere. The D17 cells (5.0 × 10^5) were seeded in 50-mm cell culture dishes and incubated for 24 hours. Cells were then incubated for 6 hours with PBS solution (0.1% volume) or desferrioxamine at a concentration of 150 μM. Cells were then incubated with 10, 25, 50, or 100 μM of dihydroartemisinin or DMSO (0.1% volume [control wells]) for 12 hours, after which cells were incubated for 1 hour with 6-carboxy-2',7'-dichlorodihydrofluorescein at a concentration of 10μM in RPMI medium and dihydroartemisinin or DMSO for 3 hours. Cells were collected, resuspended in PBS solution, and analyzed by use of flow cytometry. As a control treatment for generation of ROS, D17 cells were incubated for 1 hour with 500μM hydrogen peroxide and 6-carboxy-2',7'-dichlorodihydrofluorescein at a concentration of 10μM in RPMI, with or without the addition of 0.1% DMSO. Medium was removed, cells were rinsed with PBS solution, and cells were then incubated in fresh RPMI medium containing dihydroartemisinin or DMSO for 3 hours. Cells were collected, resuspended in PBS solution, and analyzed by use of flow cytometry. As a control treatment for generation of ROS, D17 cells were incubated for 1 hour with 500μM hydrogen peroxide and 6-carboxy-2',7'-dichlorodihydrofluorescein at a concentration of 10μM in RPMI, with or without the addition of 0.1% DMSO. Medium was removed, cells were rinsed with PBS solution, and cells were then incubated in fresh RPMI medium containing hydrogen peroxide or DMSO (or both) at the same concentrations for an additional hour. Experiments were repeated 2 times.

**Statistical analysis**—Changes in cell viability (measured by use of the nucleic acid fluorescence assay kit and cytoplasmic nucleosomes [measured by use of the ELISA]) were compared by use of a 1-way ANOVA. A pairwise multiple comparison procedure was performed by use of the Dunnet test. The proportions of cells in sub G1, G0/G1, S, and G2/M phases of the cell cycle in the control and treated groups at various time points were compared statistically with contingency tables by use of the χ² test. All statistical analyses were performed by use of commercially available software. Statistic significance was established as values of P ≤ 0.05.

**Results**

Dihydroartemisinin decreased cell viability in all 4 canine osteosarcoma cell lines in a dose-dependent manner (Figure 1). A significant decrease in cell viability was detected at concentrations of 5, 50, 10, and 10μM for D17, OSCA2, OSCA16, and OSCA50 cell lines, respectively. Calculated IC₅₀ values for the cell lines were 8.7, 43.6, 16.8, and 14.8μM for D17, OSCA2, OSCA16, and OSCA50, respectively. Microscopic examination revealed cellular fragmentation and loss of attachment to the cell culture plate in all 4 cell lines (data not shown). In contrast, DMSO-treated (control) cells had no evidence of cytotoxicosis.

The mode of dihydroartemisinin-induced cell death was assessed by use of an ELISA-based technique for detection and quantitative determination 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 thus represent a quantitative measure of this process. Dihydroartemisinin treatment induced a dose-dependent increase in formation of free cytoplasmic nucleosomes (Figure 2). A significant increase in cytoplasmic nucleosomes was detected at a concentration of 10μM for the D17, OSCA16, and OSCA50 cell lines and at a concentration of 40μM for the OSCA2 cell line. This was accompanied by cleavage of caspase 3 (Figure 3). Considered together, these data indicated that the anti proliferative effect of dihydroartemisinin was attributable, at least in part, to apoptosis of treated cells.

Effects of dihydroartemisinin on cell-cycle progression were evaluated. An increase in the proportion of cells in the sub G1/G0 phase was detected in all 4 cell lines after incubation with dihydroartemisinin, which suggested induction of apoptosis (Figure 4; Table 1). Dihydroartemisinin treatment also resulted in an increase in the proportion of G2/M phase populations and a decrease in the proportion of G1/G0 phase populations for all 4 cell lines.

Finally, to determine whether dihydroartemisinin cytotoxic effects in canine osteosarcoma cells reflected a hypothesized iron-mediated generation of ROS, induction of ROS was measured in the D17 cell line. The 6-carboxy-2',7'-dichlorodihydrofluorescein is a cell-permeating nonfluorescent probe that fluoresces after oxidation by ROS, which can be measured by use of flow cytometric analysis. Thus, an increase in fluorescence indicated an increase in the amount of ROS. Dihydroartemisinin induced a dose-dependent increase in generation of ROS in D17 cells (Figure 5). This increase in ROS was detected at concentrations of ≥ 5μM (D17), 10μM (OSCA16 and OSCA50), and 50μM (OSCA2) dihydroartemisinin, compared with values for 0μM dihydroartemisinin.
Figure 2—Mean ± SD formation of cytoplasmic nucleosomal DNA in canine osteosarcoma cell lines D17 (A), OSCA2 (B), OSCA16 (C), and OSCA50 (D) after incubation with various concentrations of dihydroartemisinin for 48 hours. Results were similar when the experiment was repeated. *A significant (P = 0.01) increase in cytoplasmic nucleosomes was detected at concentrations ≥ 10µM for the D17, OSCA16, and OSCA50 cell lines and at 40µM for the OSCA2 cell line, compared with values for the control treatment (0.1% volume DMSO).

Figure 3—Western immunoblot for cleavage of caspase 3 in 4 canine osteosarcoma cell lines incubated for 48 hours with 10 or 20µM dihydroartemisinin (DHA) or DMSO (control treatment). After incubation, protein lysates were collected and then analyzed by use of western immunoblots with antibodies specific for caspase 3 and β-actin.
was significantly suppressed by prior incubation of cells with the iron chelator desferrioxamine, which is consistent with results of other studies regarding the role of iron in cytotoxic effects of artemisinin. Hydrogen peroxide, an established inducer of ROS, also was able to induce generation of ROS in D17 cells in vitro.

**Discussion**

During the past 2 decades, the antimalarial agent artemisinin and its derivatives have attracted substantial interest as potential novel anticancer agents, cancer preventatives, multidrug-resistance–reversal agents, and radiosensitizers. Artemisinin-derived
1,2,4-trioxanes have substantial activity at concentrations in the nanomolar to micromolar range against a number of human cancer cell lines.3–10,12–20 The biological activity in human cancer cell lines and apparently low toxicity for human malaria patients have stimulated the use of artemisinin by owners for the treatment of dogs with various malignancies (particularly osteosarcomas) because the drug is readily available as an over-the-counter product. However, no investigators have conducted a study to critically evaluate the potential in vitro or in vivo activity of artemisinin against canine cancer cells.

In the study reported here, a dose-dependent decrease in the viability of osteosarcoma cell lines was detected after exposure to dihydroartemisinin. The IC_{50} values for these cell lines ranged from 8.7 to 43.6μM. These values are similar to those reported27 for exposure of the National Cancer Institute 55 human cancer cell line panel to 3 artemisinin derivatives (ie, artemether, and arteether). For that panel, the greatest susceptibility was detected in non–small-cell lung cancer cell lines (mean IC_{50}, 26μM artemunate).17 Unfortunately, no sarcoma cell lines were tested.

In our study, we also detected a dose-dependent increase in cytoplasmic nucleosomes and accumulation of cleaved caspase 3, which is consistent with dihydroartemisinin-induced apoptotic cell death. Furthermore, there was a dose-dependent increase in the sub G_{0}/G_{1} phase population in treated cells, which provided additional support that the mechanism of cell death for dihydroartemisinin is attributable, at least in part, to apoptosis. Induction of apoptosis by artemisinin derivatives has been reported in other studies31,32 in which investigators evaluated human cancer cell lines. A decrease in the proportion of cells in the G_{0}/G_{1} phase was evident in the cell-cycle analysis of dihydroartemisinin-treated cells. The underlying mechanism for this alteration in cell-cycle phase distribution is unknown.

One of the postulated mechanisms of artemisinin- and dihydroartemisinin-induced cytotoxicosis is iron-mediated generation of ROS, which is evident for antimalarial activity. Both antimalarial and tumoricidal activities of artemisinin are iron-dependent events.36,39 For example, the in vitro cytotoxic effects of dihydroartemisinin in papilloma virus–infected epithelial cells can be reversed by chelating iron from the culture medium.26 To confirm that there was generation of ROS in canine osteosarcoma cells, we measured ROS activity in the D17 cell line after incubation with dihydroartemisinin. As expected, the generation of ROS by dihydroartemisinin was evident in a dose-dependent manner. Generation of ROS was completely inhibited by addition of desferroxamine (an iron-chelating agent) to the culture medium, which indicated that generation of ROS by dihydroartemisinin also is an iron-dependent event in canine cells.

The intrinsic oxidative stress of cancer cells is a feature that can be exploited therapeutically. In addition to artemisinin and its derivatives, a number of commonly used chemotherapeutic agents, including procarbazine, anthracyclines, cisplatin, bleomycin, and ionizing radiation, exert at least a portion of their killing effect on cancer cells through the production of free radicals.31,32 Because cancer cells generate large amounts of ROS and are more dependent on antioxidant enzymes than are normal cells, it would suggest that inhibition of antioxidant enzymes or exposure to additional amounts of exogenous ROS may offer a unique therapeutic opportunity. Other compounds that increase generation of ROS that are being investigated include arsenic trioxide, emodin, bortezomib, histone deacetylase inhibitors, and N-(4-hydroxyphenyl) retinamide.31,33

Analysis of the data for the study reported here indicated that dihydroartemisinin-induced apoptosis of canine osteosarcoma cell lines required drug concentra-
tions in the micromolar range. Peak plasma concentrations after an antimalarial dose of orally administered dihydroartemisinin (4 mg/kg) in human malaria patients range from 1.9 to 16 μM (median, 4 μM).

These values are lower than the cytotoxic range for our study; however, the low amount of toxic effects for artemisinins in human patients and the extremely high LD₅₀ values of artemisinin derivatives in another study in rats and mice suggest that cytotoxic plasma concentrations could be achievable in vivo by use of higher doses of artemisinin derivatives than those currently being empirically used in dogs.

Analysis of our data indicated in vitro biological activity of dihydroartemisinin against multiple canine osteosarcoma cell lines. Additional studies are warranted to evaluate the in vivo safety and efficacy of artemisinin and its derivatives in dogs with osteosarcoma.

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