Safety and Efficacy Field Study of Artesunate for Dogs with Non-resectable Tumours

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Abstract. *The anti-malarial drug artesunate has shown anticancer activity in vitro and in preliminary animal experiments, but experience in patients with cancer is very limited. Pre-clinical studies in dogs indicated morbidity at high dosage levels. This study evaluated the effects of artesunate in canine cancer cell lines and in canine cancer patients. Four canine cell lines were tested in vitro for sensitivity towards artesunate and dihydroartemisinin (DHA; active metabolite of artesunate). The half-maximal inhibitory concentration (IC_{50}) values for artesunate or DHA were 2-60 μM in three cell lines, while one cell line was much less sensitive to artesunate (IC_{50} 337 μM) than to DHA (IC_{50} 50 μM). A safety/efficacy field study with artesunate was conducted in 23 dogs with non-resectable tumours. Artesunate was administered for 7-385 days at a dosage of 651-1178 (median 922) mg/m^2. No neurological or cardiac toxicity was observed and seven dogs exhibited no adverse effects at all. Fever and haematological/gastrointestinal toxicity, mostly transient, occurred in 16 dogs. One dog died from pneumonia. Plasma artesunate and DHA levels fell below the limit of detection within 8-12 h after artesunate administration, while levels after two hours were close to 1 μM. Artesunate produced a long-lasting complete remission in one case of cancer and short-term stabilization of another seven cases.*

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In both human and veterinary medicine, there remains an urgent need for novel anticancer drugs with activity towards tumour types that are refractory to standard cytostatics.

Artesunate, a semi-synthetic derivate of artemisinin, has been found to be cytotoxic *in vitro* towards many human cell lines of different tumour origins (1, 2). Artemisinin-type drugs were initially described as potent anti-malaria drugs able to overcome multidrug resistance of *Plasmodium*. The mechanism of action of these compounds is based upon the formation of free radicals and the alkylation of cellular proteins, which leads to cell death (3-5). Ferrous iron enhances the cytotoxicity of artemisinin and artesunate towards *Plasmodia*, as well as towards cancer cells (2, 6, 7). In cancer cells, artesunate has also been shown to induce DNA strand breaks (8, 9), cell-cycle arrest at G_0/G_1 (10) or G_0/M (11), and to induce apoptosis both by p53-dependent and independent pathways (9, 11, 12). Several antioxidant stress genes as well as the activated epidermal growth factor receptor, confer resistance to artesunate. Yet, the effect of artesunate appears not to be diminished by genes that confer resistance to established antitumour drugs (multidrug resistance-1, multidrug associated protein-1, breast cancer resistance protein, dihydrofolate reductase, ribonucleotide reductase) (12-16). Artesunate may also inhibit the metastatic cascade by suppressing matrix metalloproteinase activity (17). The anticancer activity of artemisinin and artesunate has also been shown in human xenograft tumours in mice (18-20) and in clinical studies in individual human patients suffering from laryngeal carcinoma, and uveal melanoma (21, 22). A randomized controlled trial with artesunate combined with standard anticancer drugs in advanced human non-small cell lung cancer demonstrated elevated short-term survival and prolonged time-to-progression without

additional side-effects (23). Oral administration of artenimol-R, a succinate ester of dihydroartemisinin (DHA, the active metabolite of artesunate) improved the clinical condition and survival of 10 human patients with advanced cervical carcinoma (24). However, there is a paucity of data on the toxicity of artesunate and its derivates in humans and animals at the relatively high dose level that might be necessary for antitumour effects.

Like in human medicine, several canine tumour types lack satisfactory treatment options. This problem can be approached in a constructive way, through the use of pet dogs as relevant animal models for human diseases and, while adhering to ethical guidelines, by extracting data from veterinary studies that contribute to the safe implementation of new therapies in human patients (25).

The aim of the present study was to make a first assessment of the safety and efficacy of artesunate in a field study of dogs with selected, spontaneously-occurring, non-resectable tumours. Four types of non-resectable malignant tumours were chosen for this trial: Histiocytic sarcoma (HS), oral squamous cell carcinoma (SCC) shown to be unresponsive to Cox2 treatment (meloxicam), and oral melanoma, as well as chemotherapy-resistant malignant lymphomas. This selection of cancer types was based upon the relatively frequent admission of such cases to oncology units, a poor responsiveness to classical cytostatic drugs and, based upon type and location, poor prospects or sheer impossibility of surgical resection.

HS represents a frequently occurring tumour of joints or subcutaneous soft tissue in some canine breeds. Even if resection is possible, it is often highly mutilating and most affected dogs die within one year (26). Lomustine-based chemotherapy has shown some activity leading to an overall median survival of 107 days (27). Oral SCC represents another common tumour type in dogs, with size and location often impeding surgical resection. Some advances have been made in SCC by using Cox2 inhibitors, but response rates are less than <20% (28). The location and extent of most oral melanomas also makes resection practically impossible. Even with resection, most animals die within one year due to local and distant recurrences, and sensitivity to chemotherapy is poor (29), similar to metastatic types of melanoma in humans (30). Malignant lymphoma, which is comparable to human non-Hodgkin's lymphoma, is responsive to cytostatic treatment in about 75% of dogs, but drug resistance develops in the majority of cases within one year after diagnosis (31).

In addition to the clinical pet study, four immortalized canine cancer cell lines were analyzed *in vitro,* to examine whether the cytotoxicity of artesunate and DHA seen with human cancer cell lines can be compared to those originating from canine tumours. For canine subjects, great care was taken to minimize the risk of neurotoxicity and mortality, effects seen after intramuscular application of some artemisinins (32, 33). To minimize these and other toxicities, which were reported at artesunate dosages of 1600 mg/m^2, a maximum dosage of 1200 mg/m^2, recalculated from body weight in the original publication (34), was chosen and achieved by stepwise daily increased oral dosages in as far as this dosage was tolerated. Plasma artesunate and DHA levels were analyzed for 12 of the 23 canine patients.

Unpublished preliminary toxicity data of the current study have been used in the set-up of a trial of metastatic breast cancer in humans (www.clinicaltrials.gov NCT00764036).

This study was presented in a preliminary form as poster at the 21st European College of Internal Veterinary Medicine congress, Sevilla, September 8-10, 2011.

Materials and Methods

Drug. Artesunate (Arinate®) was obtained from Dafra Pharma R & D (Turnhout, Belgium) as powder of high-grade purity for the *in vitro* assays and as 200 mg tablets for the *in vivo* study.

Cell lines and cell culture conditions. A panel of four canine cancer cell lines were chosen to test for *in vitro* sensitivity to tumouricidal effects of artesunate and DHA. The cell line CMTU335 was isolated from a mammary osteosarcoma (35). P114 was isolated from a highly malignant anaplastic mammary carcinoma (36). These two cell lines were grown in Dulbecco's Modified Eagle Medium (Invitrogen NV, Leek, the Netherlands) supplemented with 10% v/v fetal bovine serum (FBS) (Harlan Sera-Lab Ltd., Loughborough, UK) with standard antibiotics (penicilline-streptomycine; Gibco/Invitrogen, Breda, the Netherlands) at 37˚C in a humidified atmosphere of 5% CO_2 in air. In addition, two cell lines from canine histiocytic malignancies (Nike and 030210, isolated and characterized by Dr P.F. Moore, University of California, Davis, CA, USA) were studied (37). These cell lines were cultured in RPMI-1640 supplemented with 10% v/v FBS, 1% v/v HEPES, 1% v/v sodium pyruvate, 1% v/v non-essential amino acids, and 1% Glutamax with standard antibiotics (penicilline-steptomycine) at 37˚C in a humidified atmosphere of 5% CO_2 in air, and examined for sensitivity towards tumouricidal effects of artesunate or DHA.

Cell growth assay. Growth rate experiments were performed in triplicate. For the first two cell lines, cells were seeded at the density indicated in Table I into each well of a 96-well plate (Becton Dickinson, Breda, the Netherlands). DHA (Sigma-Aldrich, Zwijndrecht, the Netherlands) or artesunate (Dafra Pharma R & D) was dissolved in DMSO, the latter reaching a final concentration of 0.1% in the cultures, and diluted in culture medium with serum to concentrations of 0 to 500 μM. Cells were incubated with either drug for 24 h. After treatment 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldridge) was added to a final concentration of 0.5 mg/ml and cells further incubated for 2-4 h. Cell culture media were removed and cells were lysed in 100% DMSO and the absorbance of the dissolved formazan was measured at 595 nm and corrected for background absorbance at 650 nm using a microplate reader (BioRad, Model 3550, Veenendaal, the Netherlands). In the second *in vitro* experiment, cells of the two HS

Table I. *The half-maximal inhibitory concentration (IC_{50}) values by cell growth assay in canine cell lines treated in vitro for 24 hours with dihydroartemisin (DHA) or artesunate (ART)*

Cell line	Histogenic origin	Cells seeded (n)	Assay	IC_{50} (μM)	
				DHA	ART
CMT3-U335	Mammary sarcoma	4.10^3	MTT	60	55
P114	Mammary carcinoma	1.10^4	MTT	50	337
030210	Histiocytic sarcoma	1.10^5	XTT	39	20
Nike	Histiocytic sarcoma	2.10^5	XTT	2.0	2.3

cell lines were seeded at the indicated density (Table I) based upon their growth characteristics. Since these HS cell lines demonstrated growth in suspension to varying extents, the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay instead of the MTT assay was chosen to test growth inhibition. After 24 h of incubation with DHA or artesunate (see above) XTT (Sigma-Aldrich) was added to a final concentration of 0.2 mg/ml together with its activation reagent for a further incubation of 2-4 h. Subsequently, the absorbance of the dissolved formazan was measured at 450 nm with 650 nm as reference wavelength. The 50% inhibitory concentration (IC_{50}) was calculated from the dose-response curves by 4-parameter logistic curve fitting (Sigma plot 11, San Jose, Ca, USA), in each case.

Design of safety study of artesunate in non-resectable canine tumours. All dogs were enrolled (2006-2009) in the clinical study either in Utrecht or in Wageningen by one of the authors (GRR) after selection based on tumour type and extension. Owners accepting study conditions provided written consent for their dogs to participate in the study. Dogs suffering from non-resectable HS, oral SCC that were not responsive to high-dose Cox2 inhibition with meloxicam (MetacamR, Boehringer-Ingelheim, Ingelheim, Germany; at 0.3 mg/kg per day), or melanoma were included in the study based on cytological and histological examination. Confirmation of HS was based on positive immunoreactivity for (CD18) in histological sections (26). Three dogs with chemotherapy-resistant malignant lymphoma (ML) but good clinical performance were also included in the trial. The Medical Ethics Committee of The Utrecht University Department of Clinical Sciences of Companion Animals had approved the study design (project # DE1200532021).

All cancer cases chosen for the study had to be at a clinical performance ranking of 0-II (38), and cumulative mass (diameter) of the all tumour foci had to be within the limit that produces a clinical estimate for survival of at least one month. As such, the diameter of melanoma and SCC was set at a limit of 4 cm, while for HS (with or without internal organ involvement) and ML, the cumulative diameter was set at a limit of 15 cm. Other selection criteria included sufficient function of critical organs: haematocrit: >0.30 l/l; creatinine <91 + 1.2 × body weight in kg, in μmol/l; bile acids <25 μmol/l.

Dosage schemes. Artesunate was administered orally divided over two daily dosages. Dosage levels during the first five days increased stepwise from 600 to 1000 mg/m^2/day and then were maintained at this level till day 7-14, depending on the presence of adverse effects and then, once considered justified, increased to 1200 mg/m^2 during the remainder of the treatment period. In case of minor adverse effects such as reduced appetite, the owner was instructed to lower the dosage by 25% for one to two days and then to try return to the planned dosage. In the case of severe and/or continued adverse effects, the owner was instructed to withhold treatment and to have the animal symptomatic treatment provided, such as administration of anti-emetics in case of nausea or of antibiotics in case of fever. In such dogs, and after normalization of the dog's condition, the subsequent dosage was set at 800 to 1000 mg/m^2. In essence, adverse events of grade 2 or higher following the criteria set forth by the Veterinary Cooperative Oncology Group (39) led to a dosage reduction or short-term halt of artesunate administration (one to two days; in one dog #23 for one week). Every treatment period was followed by complete physical and haematological examination at 2, 4, 6, 8 weeks and every two months thereafter, and subsequent continuation of treatment in cases without major tumour progression (>30% mass increase). Continued treatment in some cases was accompanied by a dose reduction, which was decided based on the presence and severity of adverse effects. At the completion of treatment, the mean effective dosage (MED) was calculated as the cumulative daily dosage per square metre divided by the number of treatment days.

Measurement of response to treatment. At the start of treatment, all dogs were examined by physical examination and radiographic examination of the thorax (melanoma, HS, SCC) plus ultrasonographic examination of the abdomen (ML, melanoma, HS) as well as complete haematological and chemical blood examination. All dogs had externally located tumour mass(es). All dogs with HS were affected by the localized soft tissue form, while only in four dogs with HS, limited metastasis (cumulative diameter <4 cm) was recognized in internal organs. Diameters of visible/palpable tumors were measured in three directions at every physical examination and recorded. In addition and depending upon type of response (not for progressive disease) repeated radiographic/ultrasonograpic examination was performed at 6-8 weeks and at two months intervals for one year, the latter accomplished only in the dog that reached long-lasting remission. Diameters of internal tumour masses were measured in two directions by radiography/ultrasonography. The response criteria were complete response (CR), disappearance of any measurable tumour; partial response (PR), decrease in total tumour volume of >50%, provided no new lesions or progression from any lesion developed; stable disease (SD), decrease <50% of tumour volume and no increase >25% of any measurable tumour; progressive disease (PD), 25% or more increase in size of any measurable lesion

or appearance of new lesions. Response criteria follow the guidelines for treatment response evaluation of solid tumors as in use at the time of the clinical study (2006-2009) (40) or for canine malignant lymphoma (41).

Measurement of plasma concentrations of artesunate and DHA. For dogs treated in Utrecht, venous blood samples were collected in EDTA before or during treatment with artesunate and immediately processed by centrifugation at 4˚C for storage at –70˚C. The approximate time interval between the last artesunate administration and blood collection was recorded. Remaining EDTA-blood obtained for diagnostic procedures in five non-trial dogs were analyzed to examine for possible background values of the assays. Plasma levels of artesunate and DHA were determined by high-performance liquid chromatography-mass spectrometry, as described elsewhere (42). For dogs treated in Wageningen immediate storage at –70˚C was not possible and these cases were excluded from the analysis of drug plasma concentrations.

Statistics. The Wilcoxon signed rank-test was used for analysis of variation in group medians of numerical variables before and during treatment. ANOVA was used to test the interdependency of numerical variables. Variation among groups of categorical variables was assessed with the Fisher's exact test. *p*-Values <0.05 were considered significant.

Results

In vitro cytotoxicity assays. Artesunate and DHA reduced the viability of cells of the four canine cell lines in a dose-dependent manner. The IC_{50} values for DHA ranged from 2.0-60 μM (Table I), with similar results obtained by using artesunate in three cell lines, but one cell line (P114) was much less sensitive to artesunate than to DHA (IC_{50} 337 and 50 μM, respectively).

Field study of artesunate in canine cancer. A total of 23 dogs were included in the artesunate trial between 2006 and 2009, of which 12 were diagnosed with HS, three with oral melanoma, five with SCC and three with chemotherapy-resistant malignant lymphoma. In one dog (#13) initially diagnosed with oral SCC, the clinical course prompted new biopsies, leading to a revised diagnosis of dental root infection with granuloma. In this dog, only the incidence of adverse effects were recorded during artesunate treatment (but none occurred) and the dog was excluded from further study at the time of this revision. The clinical data for all animals are listed in Table II. The variation in artesunate dosage levels due to dose adaptations following the occurrence of adverse effects led us to calculate the MED level per animal per square metre during the period of treatment. As a result artesunate was calculated as having been administered in a MED range of 651-1178 (median 992) mg/day for 7-385 (median 28) days.

Adverse events. Seven dogs underwent treatment with no visible toxicity. Five animals developed fever (Table II). One out of these five died from septic pneumonia and calcinosis of the lungs, as shown at autopsy. The latter state was attributed to previous long-term existence of hypercortisolism, which was considered not to be completely under control by suppressive treatment, and was likely a predisposing factor for this fatality. In one of the other four dogs, a blood sample for culture was obtained during an episode of fever, and this sample tested positive for bacterial infection (*Micrococcus* sp.). Recovery occurred soon after administration of broad-spectrum antibiotics in this animal, as well as in the other dogs with fever. Gastrointestinal toxicity was observed in nine dogs (three with grade 1, five with grade 2 and one with grade 3). Neurological toxicity symptoms were not observed in the treated dogs, nor were signs of cardiotoxicity found at physical examination. Haematotoxic effects of artesunate treatment were frequently observed. A decrease in haematocrit value of 5-30% was found in 17 out of 21 dogs (Wilcoxon signed rank test: $p<0.001$), compared to levels before treatment. In four dogs, the haematocrit fell to values below 0.30 l/l, a condition considered to be significantly anaemic. In one dog, the lowered haematocrit related to the occurrence of immune-mediated haemolysis (Coombs test positive). The artesunate dosage as MED/day was not related to the extent of change of the haematocrit (ANOVA: $p=0.079$) in the whole study group nor in the group after elimination of two cases with leukaemic progression (ANOVA: $p=0.11$). Leukopenias were not seen in any dog before or during treatment. Overall, 10 dogs developed grade 2 or higher adverse events. Artesunate dosage (MED) comparing <median and ≥median was not related to the occurrence of toxicities of grade 2 or higher (Fisher's exact test: $p=0.72$)

Antitumour effects. Out of the 21 dogs with confirmed cancer that could be evaluated for response to artesunate administration, excluding #13 and #17 (Table II), one dog had a CR of its tumour at one month and was still tumour-free 14 months after the treatment had stopped (treatment was originally provided for 61 weeks). Seven animals had SD for at least four weeks (one case examined at day 25). Re-evalutation according to RECIST criteria (41) did not lead to a change in the type of response, as based upon the size and number of primary or secondary tumor lesions. Artesunate dosage (MED) comparing <median and ≥median was not related to positive treatment response (CR+SD, Fisher's exact test: $p=0.24$).

Blood levels of artesunate and DHA. The assays for artesunate or its metabolite DHA were negative in plasma samples from the five control dogs and twelve dogs tested before the first administration of artesunate. For treated animals, the time interval between the last administration of artesunate and blood collection varied greatly, either due to temporary drug withdrawal by the owner in the presence of adverse effects, or due to the time of day of the physical examination. Plasma levels of artesunate and DHA were found to have fallen below

Table II. *Effects of artesunate administration to dogs with tumours*.

Dog no.	Age (years)	Gender	Body weight (kg)	Tumour type	Duration of therapy (days)	Dose MED/m^2/day (mg)	Toxicity*	Change of haematocrit (%)	Response
01	10	M	38	HS	16	780	No	–0.08 (20%)	PD at day 16
02	2	F	56	HS	430	651 (D1-85) 548 (D86-430)	F (1; 2 d)	–0.07 (17%)	CR at day 28, treatment stopped at 61 weeks, healthy 14 months later
03	10	M	34	HS	12	857	GI (2; 2d)	0	PD at day 12
05	12	F	26	HS	13	761	GI (2; 2d)	–0.03 (8%)	PD at day 14
08	7	M	45	HS	14	738	No	–0.2(5%)	PD at day 14
09	7	F	33	HS	32	1136	No	–0.10 (20%)	SD at day 14, PD at day 32
14	9.5	F	31	HS	42	1162	GI (2; 2 d)	–0.13(30%)	SD at day 28, PD at day 42
15	10	M	46	HS	47	1031	No	–0.10 (23%)	SD at day 28, PD at day 47
18	5.5	M	42	HS	28	992	GI (1; 2d) A (1)	–0.12 (27%)	SD at day 14, PD at day 28
19	5.5	M	30	HS	14	990	GI (3; 3d) Death	ND	PD at day 14; GI-problems due to tumour progression in liver
20	8	M	30	HS	54	1031	GI (1; 3d)	–0.03 (8%)	SD at day 28, PD at day 54
23	8	M	31	HS	9+8	1020+1010	F (2; 3d; 2x)	–0.05 (15%)	PD at day 24.
04	10.5	M	36	Melanoma	25	895	F (3; 2d), death	–0.05 (9%)	SD at day 25; death at day 26‡
06	11.5	M	36	Melanoma	42	688	GI (2; 2d)	–0.05 (12%)	SD at day 14, PD at day 42
17	8	F	30	Melanoma	10	938	GI (1; 2d)	ND	Stop at week 1.5, examined at week 5; Not evaluable
07	9.5	M	37	SCC	29	676	GI (2; 2d)	+0.05 (10%)	SD at day 14, PD at day 29
10	8	M	29	SCC	40	1160	No	–0.06 (14%)	SD at day 14, PD at day 40
21	7	M	32	SCC	42	1178	A(1)	–0.13 (30%)	SD at day 28, PD at day 42
22	11.5	F	40	SCC	44	1026	F (3; 2d) A (4; 7d) Tp (3; 7d)	–0.25 (55%)	SD at day 28, PD at day 44
13	10.5	M	30	SCC#	14	990	No	+.001 (1%)	At revision no tumour
11	8	F	38	ML (resistant)	7	1062	No	+0.02 (5%)	PD at day 7
12	8	M	36	ML (resistant)	23	1055	F (1; 1d)	–0.08 (21%)$	PD at day 23
16	9	F	48	ML (resistant)	54	1015	A (2)	–0.09 (24%)$	Leukemic: SD at day 28; PD at day 54

Notes: toxicity grades (1-4) indicated as described by VCOG (reference 39). GI: Gastrointestinal, exclusively anorexia and/or vomiting; F: fever; A: anaemia, haematocrit <0.35 combined with >20% decrease of haematocrit; Tp: thrombocytopenia; HS, histiocytic sarcoma; ND, not determined; ML, malignant lymphoma; SCC, squamous cell carcinoma; MED: mean effective dosage; PD, progressive disease; CR: complete remission. ‡death due to pneumonia and calcinosis (Cushing's disease); $ decrease in haematocrit (0.39-0.31) of >20% associated with leukemic progression; #SCC based on 1st biopsy later changed into hyperplasia due to dental root abscess; no. 17: treatment was stopped at 1.5 weeks; delay of control visit till week 5 made evaluation of tumour response impossible; no. 19: adverse events ascribed to tumour progression; no. 20: osteolytic lesion in proximal humerus cytologically proven histiocytic sarcoma, with 4 cm lung mass at start of trial, radiographs at 8 weeks demonstrated PD at both sites, postmortem histology demonstrated histiocytic sarcoma at both sites; no. 22: haematological toxicity at D52: severe anaemia, thrombocytopenia and hypo-albuminaemia due to combination of immune-mediated breakdown (Coombs test positive) and GI blood loss. The dog was concurrently treated with Cox2 inhibitor (normal dosage). Treatment with artesunate and Cox2 inhibitor was stopped and corticosteroids plus antibiotics started, with recovery visible within one week; no 23: episode of grade 2 fever at day 9: withdrawal from ART treatment for 7 days; recovery on antibiotics; restart of ART on day 16 led to fever at day 24: positive blood culture (*Micrococcus* sp).

the detection limit 8-12 hours after the last administration of artesunate in all 12 animals with plasma available for analysis (data not shown). In dog #2, whose tumour showed CR, artesunate and DHA values in plasma samples collected within 2 h after artesunate administration, at different weeks during the treatment ranged from 0.28-1.30 μM and 0.73-0.94 μM, respectively. Samples obtained longer after the last artesunate administration had much lower values and a more or less time-dependent variation (Figure 1). In four other cases in which the blood samples were collected within eight hours of artesunate administration, tumour progression was observed despite the fact that levels of artesunate/DHA measured were comparable to those seen in case #2 for two of these cases (#1: 0.28 and 0.75 μM; #10: 0.63 and 0.94 μM). However, the artesunate and DHA levels were lower than for case #2 in the other two cases (#3: 0.05 and 0.04 μM, #18: 0.076 and 0.17 μM). All measurements demonstrated apparent time-dependency (not shown) after last artesunate administration.

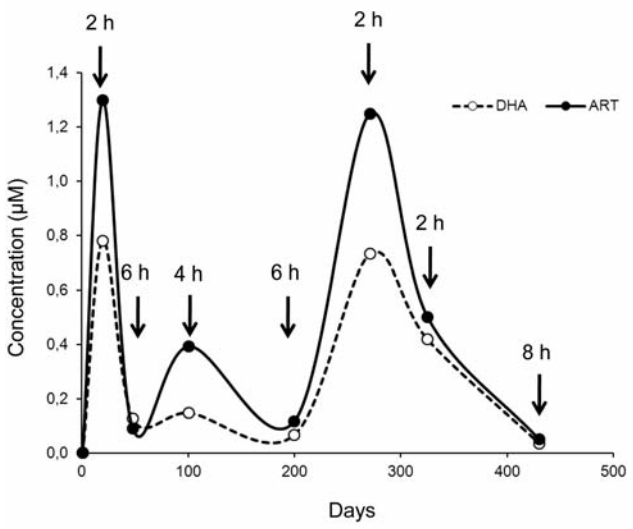

Figure 1. *Determination of plasma levels of artesunate (ART) and dihydroartemisinin (DHA) in dog #2 with complete remission. ART was administered at 950 mg divided over two daily dosages (651 mg median effective dosage (MED)/m^2) during days 1-85 and at 800 mg per day (548 mg MED/m^2) during days 86-430. Arrows indicate the number of hours from the time the last ART tablet was given until the time of blood collection.*

Discussion

The present investigation undertook a laboratory and field study of artesunate in 23 dogs with primary diagnoses of four types of non-resectable malignant neoplasms with expected or proven resistance to classical cytostatics. Firstly, we analyzed the *in vitro* cytotoxicity of artesunate and DHA in four canine cancer cell lines. The IC_{50} values for DHA in these cell lines were in the range of 2-60 μM. Another study examining the effect of DHA *in vitro* in four canine osteosarcoma cell lines, reported IC_{50} values from 9-44 μM (43). Artesunate had similar activity as compared to that of DHA in three cell lines, but one mammary tumour cell line (P114) was much less inhibited by artesunate than by DHA. Future studies should seek to establish whether this difference in sensitivity relating to lower metabolization of the parent compound. In two HS cell lines, the effects of artesunate and DHA were found to be similar. These observations suggest that most of the chosen canine cell lines are somewhat less sensitive to artemisinin derivates than are cell lines from many types of human cancer (1, 2). In a panel of 55 human-derived tumour cell lines, IC_{50} values for artesunate ranged from 1-2 μM for leukaemia and colonic cancer cell lines, *via* intermediate values for melanoma, breast, ovarian, prostate, central nervous system, and renal cancer cell lines, to relatively high IC_{50} values of ±25 μM for non-small cell lung cancer cell lines (1). In a second panel of 36 cell lines of different tumour origins, the IC_{50} values ranged between 0.512 and 124.295 μM with a mean IC_{50} value of 7.261 μM (2). For comparison, the concentrations needed to kill *Plasmodium* were in the nanomolar range (44, 45).

Since the results obtained with DHA *in vitro* in the first panel of canine cancer cell lines showed potential for treating canine cancer, we started a safety/efficacy field study in 23 dogs diagnosed with non-resectable HS, oral SCC, melanoma or drug-resistant malignant lymphoma. To the best of our knowledge, this is the first field study of artesunate with canine cancer subjects, and thus might aid in the design of future human cancer trials; at the time of writing, published human artesunate clinical trials number only two (23, 24). Dosage levels to acquire anticancer effects were expected to be higher than for anti-malarial treatment in humans (standard 50-200 mg for 3-4 days) (45) and the period of treatment was designed to be longer. Information from one company indicated that a dosage of 82.5 mg/kg body weight once daily to healthy dogs (corresponding to about 1600 mg/m^2) for four weeks produced significant morbidity but no death (34). Taking into account the possibility that dogs with cancer might be more vulnerable to adverse effects than healthy dogs, as well as the fact that the subjects were pet animals, we planned a dosage with very limited risk of mortality and low risk of high-grade morbidity. Thus, a dosage was selected of 600 mg/m^2/day, divided into two dosages, with stepwise increases until a maximum of 1200 mg/m^2/day was reached.

The adverse effects of artesunate observed in the present clinical trial included gastrointestinal toxicity, fever, and haematological toxicity in terms of a decrease in haematocrit values and anaemia. Grade 2 or higher adverse events were seen in 10 out of the 23 dogs treated with artesunate, but, with the exception of the anaemia cases, recovery was achieved in most animals by a delay or dose reduction in the artesunate treatment coupled with symptomatic treatment within a few days. It is important to note that the death of one animal due to pneumonia and calcinosis (a complication of earlier hypercortisolism) was not solely related to treatment with artesunate. However, development of septicaemia despite normal leucocyte counts can be seen as a risk of treatment, as shown by the fact that the blood culture of one of the four dogs with fever provided proof of bacteraemia. A second death that occurred during the treatment period was due to metastatic progression, as noted at necropsy. Dose reductions or delays in the administration, the latter usually for one day, were executed by all owners that noticed grade 2-4 adverse effects in their pets. In addition, other dogs were often given a reduced dosage for a shorter or longer time, with the most explicit reasons being reduced appetite (but no anorexia) or general fatigue. As a result, the MED/day was 651-1178 (median 922) mg/m^2 in the treatment group, somewhat lower than the aim at the onset of the study, but still nearly fivefold higher than that applied in human anti-malarial treatment with artesunate.

Large clinical meta-analyses of more than 15,000 human patients with malaria did not reveal significant toxicity from artesunate or related anti-malarial drugs (46, 47). In contrast, extended pre-clinical toxicity studies in healthy experimental dogs with artemisinin derivates have revealed toxic effects, leading to major concerns about severe neurotoxicity (32, 33). One of the main reasons for this striking difference is that human patients with malaria usually take tablets, while most animal toxicity studies used intramuscular application of these drugs dissolved in oil. The half-life of artemisinin-type drugs differ significantly between these oral and intramuscular applications, and the latter likely promotes accumulation of the drug (48, 49). Because oral application is also the route of choice for cancer treatment, we applied artesunate orally in the present field study. Later information justified this decision and indicated that long-term daily intramuscular administration of artesunate would have produced unacceptable side-effects, with a single injection in dogs producing muscle necrosis and inflammation (50). Whereas some toxic effects, such as haematotoxicity and gastrointestinal toxicity (34), were observed after artesunate treatment in the present study as well as in other canine studies in the literature, a striking difference was that we did not observe the severe neurotoxicity reported in many animal studies using intramuscular application (32, 33). In cats, the situation appears much more dangerous; two cats given a similar dosage on a body surface area basis, developed signs of severe neurological toxicity within 3-5 days (unpublished observation, GRR).

The observation that one out of the 21 dogs in which tumour responses could be evaluated experienced a complete and long-lasting tumour remission, while a stabilization of tumour growth was achieved in seven other dogs, for at least four weeks, indicates that it is possible to affect tumour growth using artesunate. However, the overall effect of treatment was small and further improvements to the treatment protocols are warranted, as is the extension of studies to other tumour types. It is remarkable that the dog that demonstrated a CR of its tumour (HS) was given the lowest MED and still had the highest artesunate/DHA blood levels of those dogs in which the blood concentrations were detectable. Detecting artesunate and DHA in blood was possible only for a relatively short time-period (less than eight hours) after artesunate administration. In dogs that could be evaluated, artesunate and DHA values were 1.3 and 0.94 μM, respectively, as highest values, if blood was obtained two hours after artesunate administration. These values are below the IC_{50} values determined in cell lines *in vitro*. Yet the innate capacity of artemisinin derivates to bind to cellular proteins may well lead to much higher tissue levels than those measured in the blood samples, as shown in one study in which rats were injected with radio-labelled 14C-artesunate, and plasma levels and tissue concentrations were compared at various time points. Concentrations at 1-6 h after injection were more than 100-times higher in tissue than in blood plasma, with much variation between tissues (51). Presumably a similar distribution and metabolism occurs in dogs, which may lead to effective tumour tissue concentrations of artesunate and DHA using dosages as applied in the present study to produce antitumour toxicity. Our data suggest that future studies should take into account the representativeness of cell lines studied as related to the tumours manifested in animal test subjects, and the correlation of plasma concentrations and time of drug administration with concentrations at the tissue (tumour) level.

In conclusion, the present clinical trial in dogs with cancer showed that oral administration of artesunate caused gastrointestinal and haematological toxicities, as well as fever. These toxicities are less severe than the prominent neurotoxicity observed in healthy experimental dogs treated with artemisinin-type drugs by intramuscular application. On the other hand, side-effects not reported after oral intake in human patients with malaria, but seen in the current canine cancer study may be a result of higher doses used in the latter. The frequency and severity of the adverse effects observed in this study are, however, were comparable to those observed in dogs treated with other cytostatic drugs, and were adequately managed by short-term dose reduction and symptomatic treatment. The observations from this comparative study reduce the fear of artesunate causing severe neurotoxicity and may serve to aid in designing possible future trials with artesunate for human patients with cancer.

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