Retrospective study of small pet tumors treated with *Artemisia annua* and iron

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Abstract. Artemisinin from *Artemisia annua* L. and its derivatives are well-known antimalarial drugs. In addition, *in vitro* studies, *in vivo* studies and clinical trials have demonstrated that these drugs exhibit anticancer activity in human patients with cancer. Therefore, the aim of the present study was to investigate whether a phytotherapeutic *A. annua* preparation exerts anticancer activity in veterinary tumors of small pets. Dogs and cats with spontaneous cancer (n=20) were treated with standard therapy plus a commercial *A. annua* preparation (Luparte®) and compared with a control group treated with standard therapy alone (n=11). Immunohistochemical analyses were performed with formalin-fixed paraffin-embedded tumor biopsies to analyze the expression of transferrin receptor (TfR) and the proliferation marker Ki-67 as possible biomarkers to assess treatment response of tumors to *A. annua*. Finally, the expression levels of TfR and Ki-67 were compared with the IC_{50} values towards artemisinin in two dog tumor cells lines (DH82 and DGBM) and a panel of 54 human tumor cell lines. Retrospectively, the present study assessed the survival times of small animals treated by standard therapy with or without *A. annua*. *A. annua* treatment was associated with a significantly higher number of animals surviving >18 months compared with animals without *A. annua* treatment (P=0.0331). Using a second set of small pet tumors, a significant correlation was identified between TfR and Ki-67 expression by immunohistochemistry (P=0.025). To further assess the association of transferrin and Ki-67 expression with cellular response to artemisinin, the present study compared the expression of these two biomarkers and the IC_{50} values for artemisinin in National Cancer Institute tumor cell lines *in vitro*. Both markers were inversely associated with artemisinin response (P<0.05), and the expression levels of TfR and Ki-67 were significantly correlated (P=0.008). In conclusion, the promising results of the present retrospective study warrant further confirmation by prospective studies in the future.

Introduction

Laboratory animals are indispensable for biomedical research. They are used to develop disease models to better understand the pathogenesis of human diseases and to develop novel treatment options. In cancer research, transplanted syngeneic, xenografted and orthotopic tumor models or chemically induced tumors in mice or rats have been used for decades (1). Despite these widely used experimental animal models, it has been overlooked in human oncology that animals may suffer from spontaneously arising tumors in a comparable fashion to human patients. These spontaneous animal tumors are much closely associated with the situation in humans compared with experimental tumor models, as they arise spontaneously and are neither maintained by transplantation nor are they induced by chemical carcinogens (2). Therefore, spontaneous veterinary tumors represent an attractive, although underestimated, opportunity to study novel treatment strategies prior to clinical application in human patients with cancer. As the treatment of spontaneous tumors in animals is also of importance in veterinary medicine, studies on veterinary tumors are also of great impact for veterinary oncology. The survival prognosis of malignant tumors in small animals is far from satisfactory and the majority succumb to the disease even after application of surgical, radio- or chemotherapeutic interventions (3,4). The clinical prognosis and clinical, pathological and biochemical factors influencing the survival of pets have been reported for dogs (5-9), cats (10-12), canines (13) and other species (14,15). Despite considerable progress in treating both veterinary and human tumors, the situation remains poor and numerous patients succumb to their disease. Therefore, novel treatment options are urgently required.

The majority of clinically established drugs are derived from natural products (16). Therefore, the search for novel treatments appears to be most promising when using natural sources. A recent example for the validity of this concept is

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artemisinin. Artemisinin is a bioactive terpenoid isolated from *Artemisia annua* L., which is a medicinal herb that has been used for about two millennia in traditional Chinese medicine (17). The isolation of artemisinin from *A. annua* led to a novel treatment option of eminent importance for malaria. Artemisinin and its derivatives have been responsible for the survival of millions of patients with malaria (18,19). This achievement was appreciated in 2015 with the conferment of the Nobel Prize for Medicine or Physiology to the Chinese scientist Youyou Tu (20). While artemisinin derivatives, such as artemether and artesunate, are well-established as anti-malarial drugs, herbal preparations of *A. annua* also inhibit *Plasmodia* infections in patients with malaria (21). Notably, the bioactivity of artemisinin is not restricted to malaria, and other diseases are also susceptible to artemisinin and *A. annua* treatment, such as schistosomiasis and trypanosomiasis (22-24), diverse viral infections (25) and diseases related to the metabolic syndromes, including obesity, diabetes and atherosclerosis (26-28).

Artemisinin derivatives also inhibit human tumor cell growth *in vitro* and *in vivo* (29-31). This is relevant not only for cancer therapy, but also for cancer prevention (32,33). Artemisinin derivatives exert additive or synergistic interactions in combination with a wide array of clinically established drugs (34-36). This has also been demonstrated in veterinarian tumor cell lines *in vitro* and veterinarian clinical trials (37-39). Based on the anticancer activity in experimental tumor models, it has been possible to investigate the anticancer activity in human cancer patients in the form of compassionate uses (40,41) and even to perform clinical phase I/II trials in human cancer patients (42-44). Another previous clinical phase I/II trial indicated the anticancer activity in a number of dogs with tumors (39). Preliminary results in three dogs and one cat revealed that a herbal preparation of *A. annua* (Luparte®) may exhibit the potential to prolong the survival time of animals with tumors (45). To substantiate these preliminary results from the compassionate use of *A. annua*, the present study has performed a retrospective analysis by evaluating survival times of 25 pets treated with *A. annua* compared with 11 animals without *A. annua* treatment. Independent of *A. annua* food supplementation, all animals were subjected to standard treatment protocols. In addition, the expression of two biomarkers, transferrin receptor (TfR) and the proliferation marker Ki-67, was determined by immunohistochemistry analysis of tumor biopsies.

Materials and methods

Artemisinin determination in A. annua. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 300 NMR spectrometer (Bruker Corporation). Reversed phase high-performance liquid chromatography (HPLC)-mass spectrometry (MS) analysis was performed on a Waters Alliance 2695 LC (Waters Corporation) coupled to a Quattro Ultima triple quadrupole MS (Waters Corporation) using the same separation conditions as described previously (46). The separation conditions were follow: Chromatogram column, XBridge™ column (4.6x150 mm, 5 μm); column temperature, 20˚C; and injection volume, 1 μl. Elution was performed at a flow rate of 1 ml/min, using as the mobile phase a mixture of water (A) and acetonitrile (B). The samples were eluted using the following gradient: 0 min, 98.0% A; 0-8 min, linear increase to 100% B; 100% A held for 2 min, 11-12 min, return to 98.0% A. The final optimization of ESI source operation parameters were a nitrogen gas flow of 11 l/min, nebulizer of 30 psi, capillary voltage of ±2.4 kV and a drying gas temperature of 250˚C (N2). The Quattro Ultima triple quadrupole MS operated in multiple reaction monitoring mode with a resolution of 0.7 m/z. Electrospray ionization (positive mode) and photodiode array detection at 254 nm were performed.

Different batches of *A. annua* (Luparte®) were determined by the Department of Pharmaceutical Biology, Johannes Gutenberg University (Mainz, Germany). This blinded approach was used as a quality measure to independently guarantee the presence of artemisinin in Luparte®. Each batch of *A. annua* powder (2x10 g) was extracted with two different polarity solvents, methylene chloride and methanol, at room temperature for 24 h. The extract was concentrated *in vacuo* to obtain a residue of 0.8 and 1.2 g for methylene chloride and methanol, respectively. Each extract was transferred to a small vial and kept dry at room temperature. Small crystals were formed, which were collected and washed with *n*-hexane and methylene chloride to remove extract residues. Then, ^{1}H and ^{13}C NMR analyses were performed.

Tumors. The animals were treated between 2010 and 2017. Briefly, 16 dogs (10 males and 6 females) and 4 cats (2 females, 1 male and 1 N/A) were treated with *A. annua*. The mean age of the dogs was 10.31 years and the mean age of the cats was 12.25 years. The mean weight of the dogs was 30.125 kg and the mean weight of the cats was 5.10 kg. Tissue samples were taken from all animals and initially fixed at room temperature (~22˚C) in 4% formaldehyde solution for 48 h. Following fixation, the organs were trimmed, processed and embedded in paraffin wax at approximately 60˚C. Sections were cut to a thickness of 4 μm and routinely stained with hematoxylin and eosin at room temperature. Histopathological examination of the hematoxylin and eosin-stained sections was performed under a light microscope in immersion oil with a Zeiss Axioskop. After routine pathological diagnosis, the paraffin blocks were used as excess material for subsequent immunohistochemical studies. Furthermore, the histological hematoxylin and eosin stained tumor sections were assessed for the presence of the tumor-infiltrating lymphocytes (TILs). The clinical data are presented in Tables I and II. The owners of the animals provided written informed consent for this retrospective study. The signed written consent forms are deposited at the Department of Pharmaceutical Biology, Johannes Gutenberg University (Mainz, Germany) and can be inspected upon reasonable demand.

Treatment protocol. The serum iron content was determined following collection of blood samples; a normal range is between 140 and 170 μg/dl (47). After initial iron content determination using ferrozine color test (48), the tumors were surgically removed with safety margins by a standard protocol after visual inspection and where required after lung radiography. Between blood collection and determination of the serum iron results from the clinical diagnosis laboratory, iron was administered orally [Ferrosanol® capsules

Table I. Clinical data of animals treated with *Artemisia annua* after standard treatment.

A, Carcinoma

Species	Breed	Sex	Weight, kg	Date of diagnosis and start of treatment	Tumor type	Survival time, months	Initial blood iron content, μg/dl	Treatment before *A. annua*	Age, years	Source of the tumor
Dog	Doberman	F	33	May 2, 2014	Adenocarcinoma	1.0	74	a	8	Subcutis
Dog	White Swiss Shepherd	M	32	Jan 1, 2015	Adenocarcinoma	5.0	111	a	9	Mammary glands
Dog	Pyrenean Shepherd	F	11	Apr 28, 2015	Adenocarcinoma	3.0	95	a	13	Subcutis
Dog	Poodle	Fcastr	9	Mar 17, 2011	carcinoma	48.0	134	c	14	Mammary glands
Dog	Mix	F	55	N/A	carcinoma	1.0	223	a	9	Lung
Dog	Border Collie	M	16	Oct 17, 2016	carcinoma	2.5	106	a	1	Lung
Cat	European Shorthair	Fcastr	4	Dec 21, 2012	Squamous carcinoma	5.0	45	b	16	Gingiva
Cat	European Shorthair	N/A	N/A	Sep 14, 2010	Non-specified carcinoma	38.0	222	b	12	Abdominal skin
Dog	Labrador	M	42	Aug 23, 2012	Keratinizing carcinoma	4.0	93	b	9	Nose skin
Dog	Mix	Mcastr	35	Jul 22, 2011	Hepatoma	8.0	164	a	13	Liver

B, Sarcoma

Species	Breed	Sex	Weight, kg	Date of diagnosis and start of treatment	Tumor type	Survival time, months	Initial blood iron content, μg/dl	Treatment before *A. annua*	Age, years	Source of the tumor
Cat	European Shorthair	Mcastr	5.5	Feb 21, 2014	Fibrosarcoma	0.5	66	c	10	Abdominal subcutis
Dog	Gordon Setter	Fcastr	26	Aug 24, 2010	Fibrosarcoma	72.0	85	c	10	Subcutis
Dog	Mix	M	38	Feb 04, 2010	Fibrosarcoma	13.0	107	c	13	Subcutis
Cat	European Shorthair	Fcastr	5.8	Jul 11, 2011	Fibrosarcoma	>68	84	c	11	Abdominal subcutis
Dog	Rottweiler	M	51	Nov 16, 2011	Fibrosarcoma	1.0	97	c	8	Subcutis
Dog	Mix	M	26	Jan 25, 2011	Hemangiosarcoma	>36	124	c	10	Spleen
Dog	Mix	Fcastr	27	Jan 31, 2012	Hemangiosarcoma	>18	131	c	14	Spleen
Dog	Labrador	Mcastr	33	Apr 05, 2013	Hemangiosarcoma	41.0	81	c	10	Spleen
Dog	Mix	M	22	Jan 22, 2014	Hemangiosarcoma	12.0	354	c	13	Spleen
Dog	Mix	Mcastr	26	Apr 02, 2014	Lymphosarcoma	13.0	116	c	11	Lymph node, left scapula

a, no surgical treatment done/possible; b, only incomplete/palliative surgery possible; c, macroscopic complete surgery. N/A, not available; F, female; M, male; castr, castrated.

Table II. Clinical data of animals without *Artemisia annua* treatment after standard treatment.

A, Carcinoma

Species	Breed	Sex	Weight, kg	Date of diagnosis and start of treatment	Tumor type	Survival time, months	Initial blood iron content, μg/dl	Treatment before *A. annua*	Age, years	Source of the tumor
Dog	Mix	F	6.8	Jul 22, 2011	Adenocarcinoma	N/A	6	c	12	Mammary glands
Cat	European Shorthair	Fcastr	3.8	Aug 22, 2012	Adenocarcinoma	84.0	4	c	16	Mammary glands
Dog	Labrador	Fcastr	36	Jan 21, 2011	Adenocarnoma	112.0	3	a	9	Mammary glands
Dog	Mix	Fcastr	8.6	Dec 29, 2014	Hepatoma	107.0	2	a	7	Liver
Cat	European Shorthair	Mcastr	4.2	Nov 07, 2014	Kidney carcinoma	69.0	4	c	13	Kidney

B, Sarcoma

Species	Breed	Sex	Weight, kg	Date of diagnosis and start of treatment	Tumor type	Survival time, months	Initial blood iron content, μg/dl	Treatment before *A. annua*	Age, years	Source of the tumor
Cat	European Shorthair	Mcastr	6.0	Aug 06, 2012	Fibrosarcoma	62.0	7	c	14	Abdominal subcutis
Dog	Mix	Mcastr	11.8	May 27, 2014	Hemangiosarcoma	220.0	7	c	13	Spleen
Dog	Mix	Mcastr	38.6	Sep 18, 2012	Hemangiosarcoma	136.0	10	c	13	Spleen
Dog	Dachshund	Fcastr	14.2	Jan 31, 2012	Liposarcoma	N/A	17	c	11	Eye lid
Dog	Mix	F	42.0	Aug 27, 2012	Rhabdomyosarcoma	83.0	0.1	a	11	Thigh skeletal muscles with metastases in liver and lymph nodes
Cat	European Shorthair	N/A	N/A	Apr 16, 2015	Hemangiopericytoma	80.0	16	c	13	Abdominal subcutis

a, no surgical treatment done/possible; b, only incomplete/palliative surgery possible; c, macroscopic complete surgery; N/A, not available; F, female; M, male; castr, castrated.

(100 mg/capsule or 40 mg/capsule)]. The dosage was 100 mg/30 kg BW twice daily mixed into vegetable-poor food to avoid iron binding plant molecules, such as phytane, oxalates and/or phosphates. Alternatively, iron was subcutaneously injected (Myofer®/Ursoferran®; 100 mg/ml) at a dose of 100 mg/10 kg BW daily, until the serum iron results were obtained back from the clinical laboratory. The iron substitution was individually continued until an iron content of 250±30 μg/dl (~43±5 μmol/l) was stably reached in blood serum. From the fourth day of the initial iron substitution onward, the animals were orally treated with Luparte® capsules at a dosage of 1,400 mg/m^2 body surface divided to three fractions per day (Table III). The capsules were administered 1-2 h before the next meal. The serum iron content was regularly monitored and where necessary adapted to 250±30 μg/dl.

The treatment details and the follow-up information, including survival times, have been recorded in the clinical documentation files. All ethical issues have been discussed prior to evaluation of clinical and experimental data with the *Regierungspräsidium* (Government Presidium) Freiburg (Germany). According to the design of this retrospective study, the *Regierungspräsidium* (Government Presidium) Freiburg gave written permission for the present study (Az. 35-9185.81/1, dated from February 4th, 2019).

Cell lines. The D-GBM cell line was generated by Dr George Stoica (Department of Veterinary Pathobiology, Texas A&M University, College Station, TX, USA) from a brain tumor of an 8-year-old male Boxer dog (49). The D-GBM cells used in the present study were obtained from Dr Pablo Steinberg (Institute for Food Toxicology and Analytical Chemistry, University of Veterinary Medicine Hannover, Hannover, Germany), who obtained it from Dr G. Stoica.

The histiocytic DH82 cell line was generated by Dr M.L. Wellman (Department of Veterinary Pathobiology, College of Veterinary Medicine, Ohio State University, CO, USA) (50). The DH82 cell line used in the present study was also obtained from Dr Pablo Steinberg, who purchased it from the European Tissue Culture Collection (catalog no. 94062922).

A panel of 54 human tumor cell lines of the Developmental Therapeutics Program (DTP) of the National Cancer Institute consisted of cell lines derived from leukemia, melanoma, non-small cell lung cancer, colon cancer, renal cancer, ovarian cancer, breast cancer and prostate carcinoma, as well as central nervous system tumors (51). Previously, the cytotoxicity of cells treated with artemisinin for 48 h was evaluated using a sulforhodamine B assay (52). The $\log_{10}$ IC$_{50}$ values for artemisinin and mRNA expressions for Ki-67 and TfR were available publicly in the DTP database (https://dtp.cancer.gov/default.htm).

Cytotoxicity assay. A resazurin reduction assay (Promega Corporation) was performed as previously described (53) to assess the cytotoxicity of artemisinin, artesunate and dihydroartemisinin towards the dog tumor cell lines DH82 and DGBM. The concept of the assay is based on the metabolic reduction of the non-fluorescent dye by living cells to the strongly-fluorescent dye resorufin (54). IC$_{50}$ values were calculated from dose-response curves using a non-linear regression analysis tool in GraphPad Prism 7 software (GraphPad Prism, Inc.). All IC$_{50}$ values are expressed as the mean ± standard deviation.

Each assay was performed thrice independently, with six replicates each.

Immunohistochemistry. A total of 17 tumor tissues representing different tumor types in pets were collected to evaluate the expression of Ki-67 and TfR in animal tumors (Table V). Briefly, tumor tissues were obtained from 15 dogs (7 males and 8 females) and 2 cats (both female). The mean age of the dogs was 9.33 years and the mean age of the cats was 4 years. The mean weight of the dogs was 27.266 kg and the mean weight of the cats was 4.00 kg. All the chosen animals had been kept in pet animal housing conditions. The immunohistochemical staining of tumor tissues was performed as described previously (55). The slides were washed twice with xylene (98.5% xylene for 5 min each at room temperature) to remove paraffin. Then, sample tissues were rehydrated through graded washes with isopropanol in water. Heat-induced epitope retrieval was performed using a pressure cooker as a heating device. Ultra-vision protein block and UltraVision Hydrogen Peroxide Block (catalog nos. TA-060-UB and TA-060-H2O2Q, respectively; Thermo Fisher Scientific, Inc.) were added to block endogenous proteins and endogenous peroxidase activity, respectively, to avoid non-specific background staining. Overnight incubation at 4°C was performed following the addition of monoclonal primary antibodies. For the detection of Ki-67, the SP6 clone (catalog no. ab16667; Abcam) was used at a dilution of 1:200. For the determination of TfR expression, the H68.4 clone (catalog. no. 136800; Thermo Fisher Scientific, Inc.) was applied at a dilution of 1:100. Subsequently, horseradish peroxidase-labeled polymers conjugated with secondary antibodies specific for both mouse and rabbit primary antibodies were added (catalog nos. TL-060-QPH and TL-060-QPB; Thermo Fisher Scientific, Inc.) at room temperature for 1 h, according to the manufacturer's protocol. The final staining reaction was performed with diaminobenzidine and slides were counterstained with hematoxylin for 3 min at room temperature.

The immunostaining of DH82 and DGBM cell lines was performed without the rehydration or epitope retrieval steps. Briefly, six cover slips were placed in a 6-well plate, then 5x10^5 cells/well were seeded over the cover slips and incubated in humidified 5% CO$_2$ atmosphere at 37°C overnight to let the cells attach to coverslips. Subsequently, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, and the blocking and staining steps were performed as described for the tissue section. However, the Ki-67 and TfR antibodies were diluted at 1:500 and 1:1,000, respectively. The immunostained slides were scanned using Pannoramic Desk (3DHISTECH Ltd.) and the amount of Ki-67- or TfR-expressing cells was quantified by Pannoramic Viewer software version 1.15 (3DHISTECH Ltd.).

Statistical analysis. Significance values and correlation coefficients were calculated using Pearson's correlation coefficient and Fisher's exact test with the WinSTAT software program version 2012.1 (www.winstat.com/). Linear regression was performed using Excel 2016 (Microsoft Corporation) for the $\log_{10}$ IC$_{50}$ values for artemisinin and mRNA expressions for Ki-67 and TfR. P<0.05 was considered to indicate a statistically significant difference.

Table III. Conversion table for Luparte® treatment.

Body weight, kg	Body surface, m²
0.5	0.06
1	0.1
2	0.16
5	0.29
10	0.46
15	0.61
20	0.74
30	0.97
40	1.17
50	1.36
60	1.63
70	1.7

Table IV. Correlation of Luparte® treatment and survival time.

Survival time, (months)	Treatment	No treatment
<18	13	11
≥18	7	0

P=0.033 according to Fisher's exact test.

Results

Identification of artemisinin. As presented in Fig. 1A, the ^{1}H NMR spectrum of a colorless crystal contained signals typical of artemisinin. The spectrum displayed signals for three methyl groups: Two secondary at δ_H 1.00 (d, *J*=6.5, H-14), 1.17 (d, *J*=7.2, H-13) and one tertiary methyl signal at δ_H 1.40 (s, H-15). Additionally, two aliphatic methylene signals were observed at δ_H 2.12 (brddd, *J*=14.9,4.2,2.6) and 2.40 (ddd, *J*=14.9, 13.1,4.1) for H-3a and H-3b, respectively. A characteristic downfield singlet signal at δ_H 6.01 indicated the presence of oxygenated proton for H-5. As presented in Fig. 1B, The ^{13}C NMR spectrum displayed 15 major carbon signals, including significant carbons signals confirming the presence for artemisinin as the following: δ_C 173.3 (s, C-12), 105.2 (s, C-4), 94.3 (d, C-5) and 79.5 (s, C-6) (56).

Reversed phase HPLC-MS was used to quantitatively analyze the presence of artemisinin. Fig. 1C presents the chromatogram obtained after dissolving 2 mg/ml MeOH extract, which indicated that artemisinin represents 3.46% of the *A. annua* (Luparte®) extract.

Clinical treatment. In total, 23 dogs and 8 cats were included in the present study. Among these, 20 were treated with *A. annua* in addition to their standard therapy (Table I) and 11 were subjected to standard treatment alone (Table II). The *A. annua*-treated group consisted of 10 carcinomas (8 dogs, 2 cats) and 10 sarcomas (8 dogs, 2 cats). All animals were treated between 2010 and 2017 (Table I). In the group of animals without *A. annua* treatment, 5 animals presented with carcinoma (3 dogs, 2 cats) and 6 animals had sarcoma (4 dogs, 2 cats) (Table II). The blood iron content and the survival times of all pets were recorded.

The effect of *A. annua* treatment on overall survival time was assessed. In total, 13 *A. annua*-treated animals and 11 non-treated animals exhibited a survival time of <18 months following therapy. Whereas, 7 animals in the *A. annua*-treated group had a survival time of >18 months after *A. annua* treatment, but no 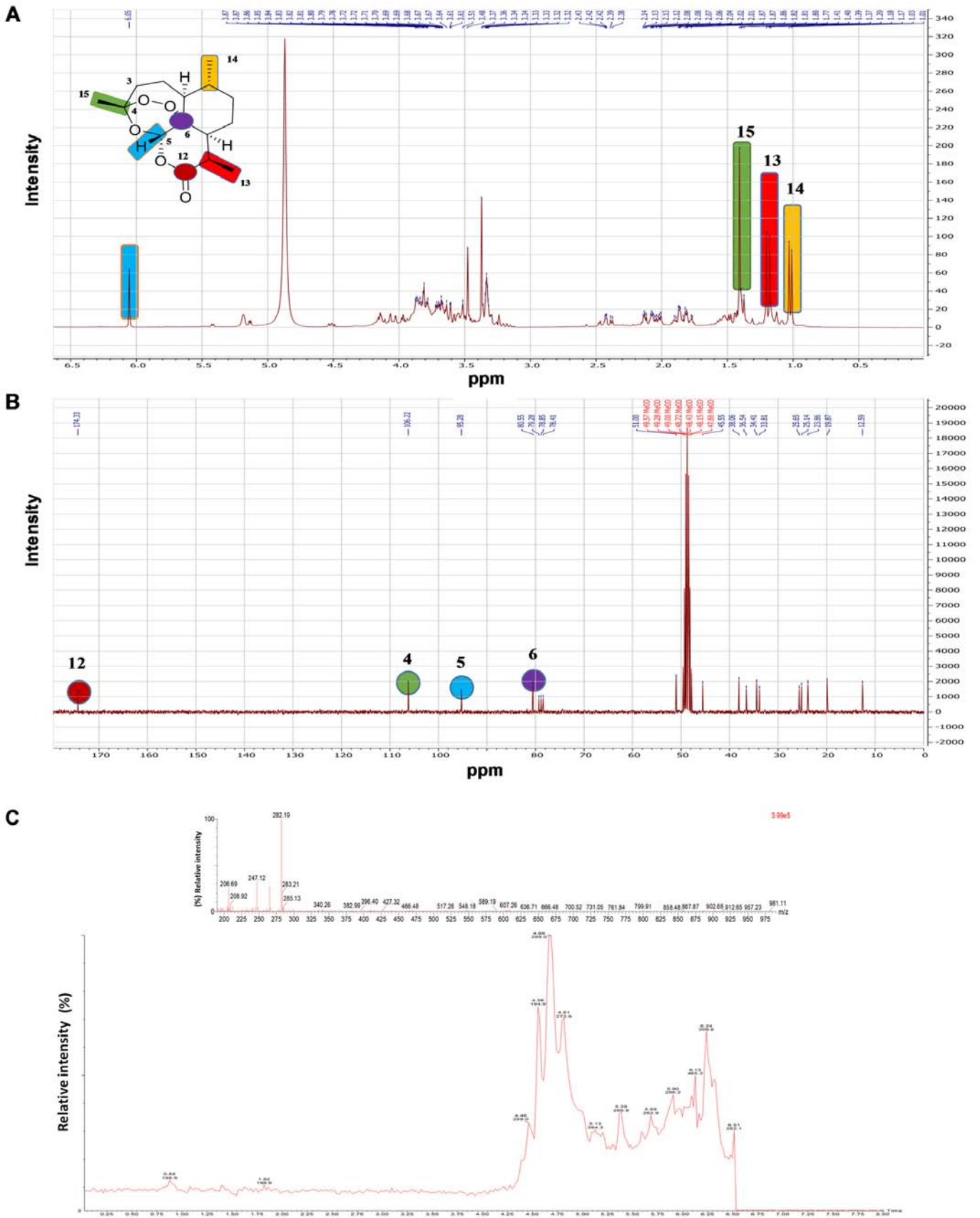animals in the non-treated group survived >18 months. This difference in survival times between the groups was statistically significant (P=0.033; two-tailed Fisher's exact test; Table IV).

Histology and immunohistochemistry. Using a second collection of 17 tumors, the histology of the tumors was determined by hematoxylin and eosin staining of formalin-fixed and paraffin-embedded tumor sections. Representative photographs of different tumor histology are presented in Fig. 2, including a tubulopapillary breast adenocarcinoma, soft tissue sarcoma, squamous cell carcinoma and neuroendocrine carcinoma.

Furthermore, immunohistochemical analyses were performed. The membrane-bound expression of TfR (CD71) and the nuclear expression of the proliferation marker Ki-67 were determined. The percentage of stained cells was quantified using a computer-based quantification system. Six different areas were selected from each tumor section to provide representative expression values. The percentage of TfR-expressing cells ranged between 43.5 (±37.9%) and 99.2% (±0.2%), whereas the percentage of Ki-67-positive cells was in a range between 6.6 (±1.8%) and 85.7% (±4.7%) (Fig. 3A and B). As tumor heterogeneity plays an important role in the response to anticancer therapy, the present study also focused on heterogeneous and homogeneous staining patterns among the different tumor biopsies. Certain tumors were very heterogeneous in the immunohistochemical staining, while others revealed uniform staining. This can be seen in the standard deviations, which ranged between 0.1 (cases no. 3, 8 and 13) and 37.9% (case 10) for TfR, and between 1.8 (case 17) and 16.3% (case 8) for Ki-67 (Table V). Subsequently, the correlation between the expression of TfR and Ki-67 with the set of tumor biopsies was investigated, which revealed a statistically significant correlation (P=0.025; r=0.469; Fig. 3C). No significant correlation was identified between blood iron content and survival time of the animals (P=0.386; r=-0.062; Fig. 3D). Notable, the association between survival time and *A. annua* treatment was significant. Pets treated with standard therapy plus *A. annua* has significantly longer survival times compared with those treated with standard therapy alone (P=0.033; Fig. 3E).

As the direct association between TfR and Ki-67 expression and the cytotoxicity of artemisinin could not be assessed in these tumor samples, the TfR and Ki-67 microarray-based mRNA expression in 54 human tumor cell lines was analyzed compared with the $\log_{10}$ IC$_{50}$ values, as determined by sulforhodamine assay. Both TfR and Ki-67 expression were significantly negatively correlated with the cytotoxicity of artemisinin in these cell lines (P<0.05; r<-0.20), which indicates a higher expression of these two markers was associated with a higher sensitivity of the cell lines to artemisinin (Table VI). The expression levels of TfR and Ki-67 were positively correlated (P=0.008; r=0.317). This panel of
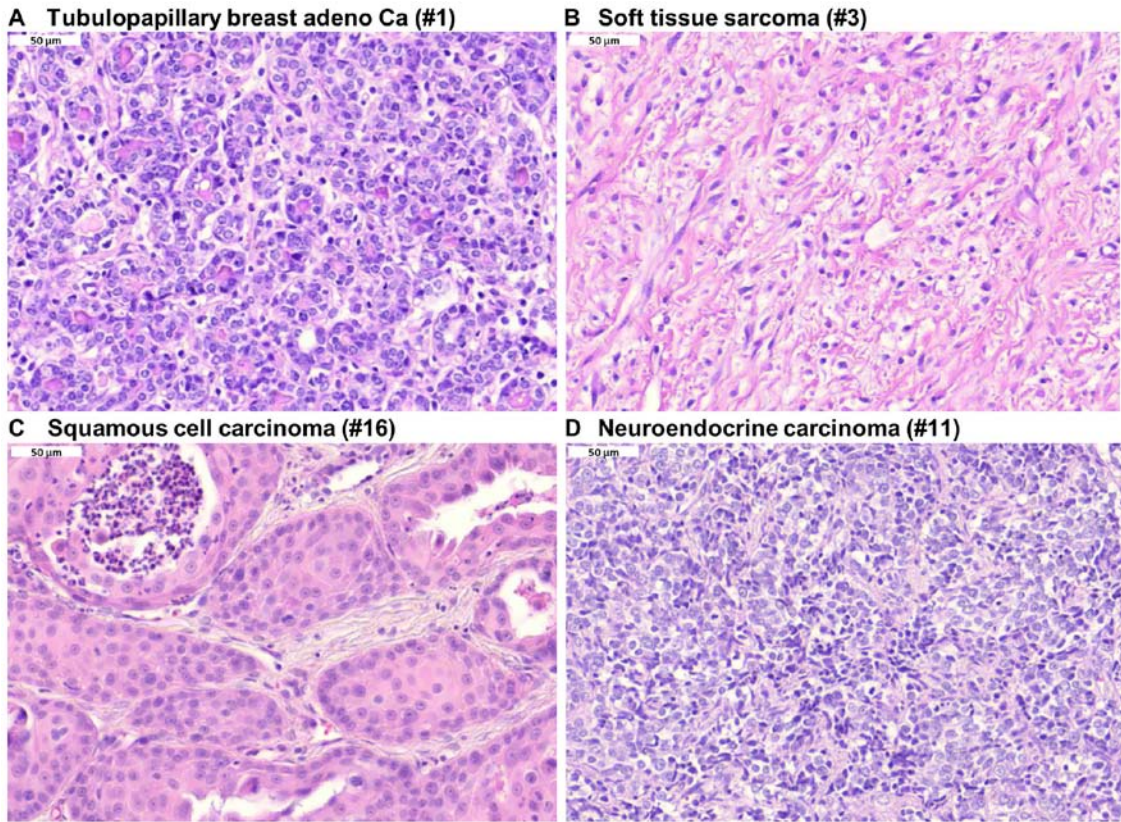
Figure 1. NMR spectra of *A. annua* chloroform extract demonstrating the presence of artemisinin. (A) ^{1}H NMR (300 MHz). (B) ^{13}C NMR (75 MHz). (C) RP-HPLC/MS chromatogram of *A. annua* (Luparte®) extract. The top figure shows the MS profile, while the bottom figure represents the HPLC measurements. NMR, nuclear magnetic resonance; RP-HPLC/MS, reversed phase high-performance liquid chromatography-mass spectrometry.

Figure 2. Hematoxylin and eosin staining of diverse pet tumors. (A) Tubulopapillary breast adenocarcinoma. (B) Soft tissue sarcoma. (C) Squamous cell carcinoma. (D) Neuroendocrine carcinoma. Magnification, x40.
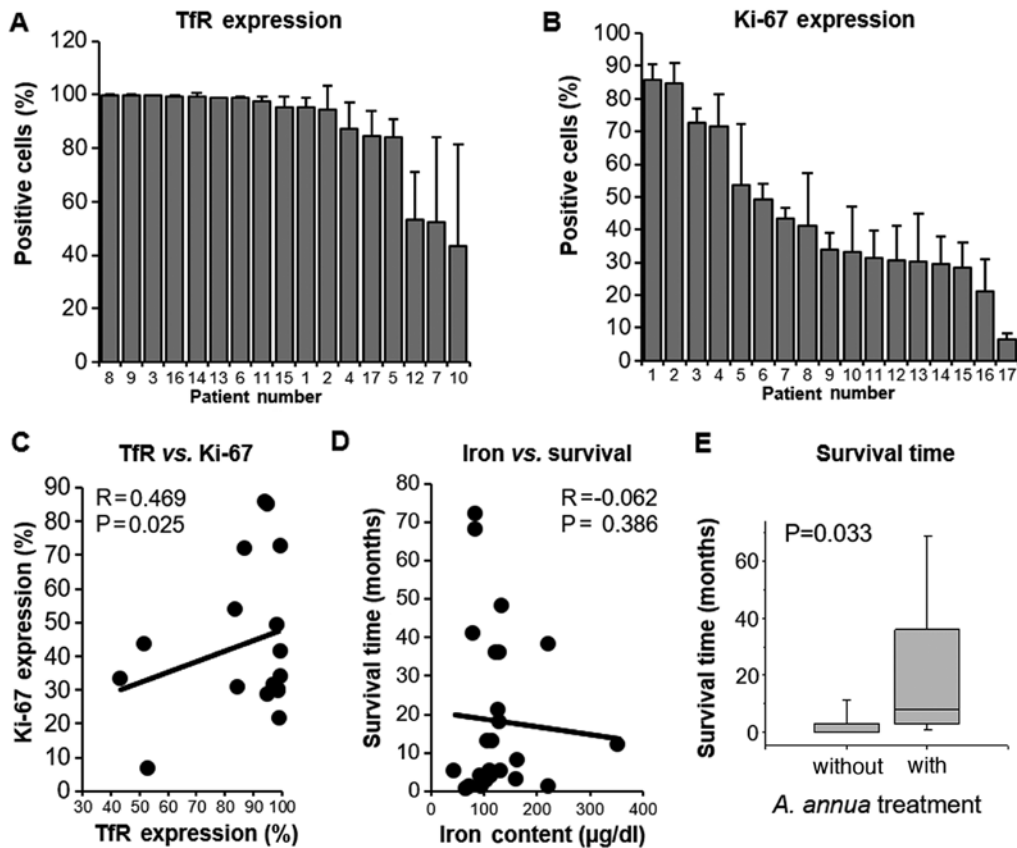
Figure 3. Quantification of immunohistochemical staining of veterinary tumors and correlation to clinical parameters. (A) TfR expression. (B) Ki-67 expression. (C) Correlation of TfR expression to Ki-67 expression. (D) Correlation of blood iron content to survival time. (E) Survival time of treated and untreated animals (P=0.033). Regression analyses were performed using Excel (Microsoft Corporation). TfR, transferrin receptor.

Table V. Expression of TfR and the proliferation marker Ki-67 in dog tumors according to immunohistochemistry.

Case	Species	Breed	Sex	Weight, kg	Age, years	Percentage of TfR-positive cells	Percentage-of Ki-76 postive cells	Histology
1	Cat	European shorthair	Fcastr	4	3	94.3±9.0	85.7±4.7	Tubulopapillary breast adenocarcinoma
2	Dog	Mix	Fcastr	17	13	95.1±3.6	84.8±5.9	Tubulopapillary breast adenocarcinoma
3	Dog	Weimarian	M	28	8	99.7±0.1	72.5±4.4	Soft tissue sarcoma, Grade 1
4	Dog	Deutsch-Adrahthaar	Fcastr	28	9	87.2±9.6	71.6±9.7	Tubulopapillary breast adenocarcinoma with lymph node metastasis
5	Dog	French bulldog	Mcastr	11	7	83.9±6.8	53.5±18.7	Soft tissue sarcoma, Grade 1
6	Dog	American Staffordshire Terrier	Mcastr	33	3	98.6±0.5	49.1±4.9	Mastocytoma, Type 2
7	Dog	Mix	M	21	12	52.1±32.1	43.5±3.1	Hemangiosarcoma
8	Dog	Podenco	Fcastr	24	12	99.9±0.1	41.1±16.3	Osteosarcoma
9	Dog	Blue lacy	M	26	11	99.8±0.2	33.9±5.0	Mastocytoma, Type 2
10	Dog	Mix	F	14	2	43.5±37.9	33.2±13.8	Mastocytoma, Type 2
11	Cat	European shorthair	Fcastr	4	5	97.4±1.8	31.3±8.3	Neuroendocrine carcinoma
12	Dog	Podenco	Fcastr	25	12	84.6±9.5	30.5±10.7	Osteosarcoma, highly malignant
13	Dog	Rottweiler	Fcastr	51	8	99.0±0.1	30.1±14.6	Adenocarcinoma metastasis
14	Dog	Mix	Mcastr	27	10	99.1±1.4	29.5±8.4	Teleangiectatic osteosarcoma
15	Dog	Labrador mix	F	37	12	95.2±3.9	28.6±7.4	Carcinoma
16	Dog	Labrador	M	43	9	99.2±0.6	21.3±9.7	Squamous cell carcinoma
17	Dog	Podenco	Fcastr	24	12	53.3±17.8	6.6±1.8	Breast carcinoma, complex/myxoid

Data are present as the mean ± standard deviation. F, female; M, male; castr, castrate; TfR, transferrin receptor.

Table VI. Correlation of artemisinin ($\log_{10}$ IC_{50} values) with microarray-based TfR and Ki-67 mRNA expression in 54 human tumor cell lines.

Marker	Artemisinin cytotoxicity	Ki-67 expression
TfR expression		
r value	-0.282	0.317
P-value	0.019	0.008
Ki-67 expression		
r value	-0.239	
P-value	0.041	

TfR, transferrin receptor.

54 human tumor lines consisted of cell lines derived from eight tumor types (leukemia, melanoma and brain tumor, and carcinoma of colon, breast, ovary, kidney and prostate). The number of cell lines of each tumor type (n=1-9) was too low to reveal significant correlations except for the three following results. In lung cancer cell lines, the $\log_{10}$ IC_{50} values of artemisinin were inversely correlated with the Ki-67 expression (P=0.009; r=-0.759). In melanoma cell lines, the expression levels of Ki-67 and TfR were significantly correlated (P=0.010; r=0.787). In renal cancer cell lines, the expression levels of Ki-67 and TfR were also significantly correlated (P=0.019; r=0.781) (data not shown). Since all other associations between artemisinin response and the expression of Ki-67 and TfR were not statistically significant, reliable conclusions on the role of Ki-67 and TfR may be drawn from the cell line panel as a whole, but not from tumor type-specific subsets.
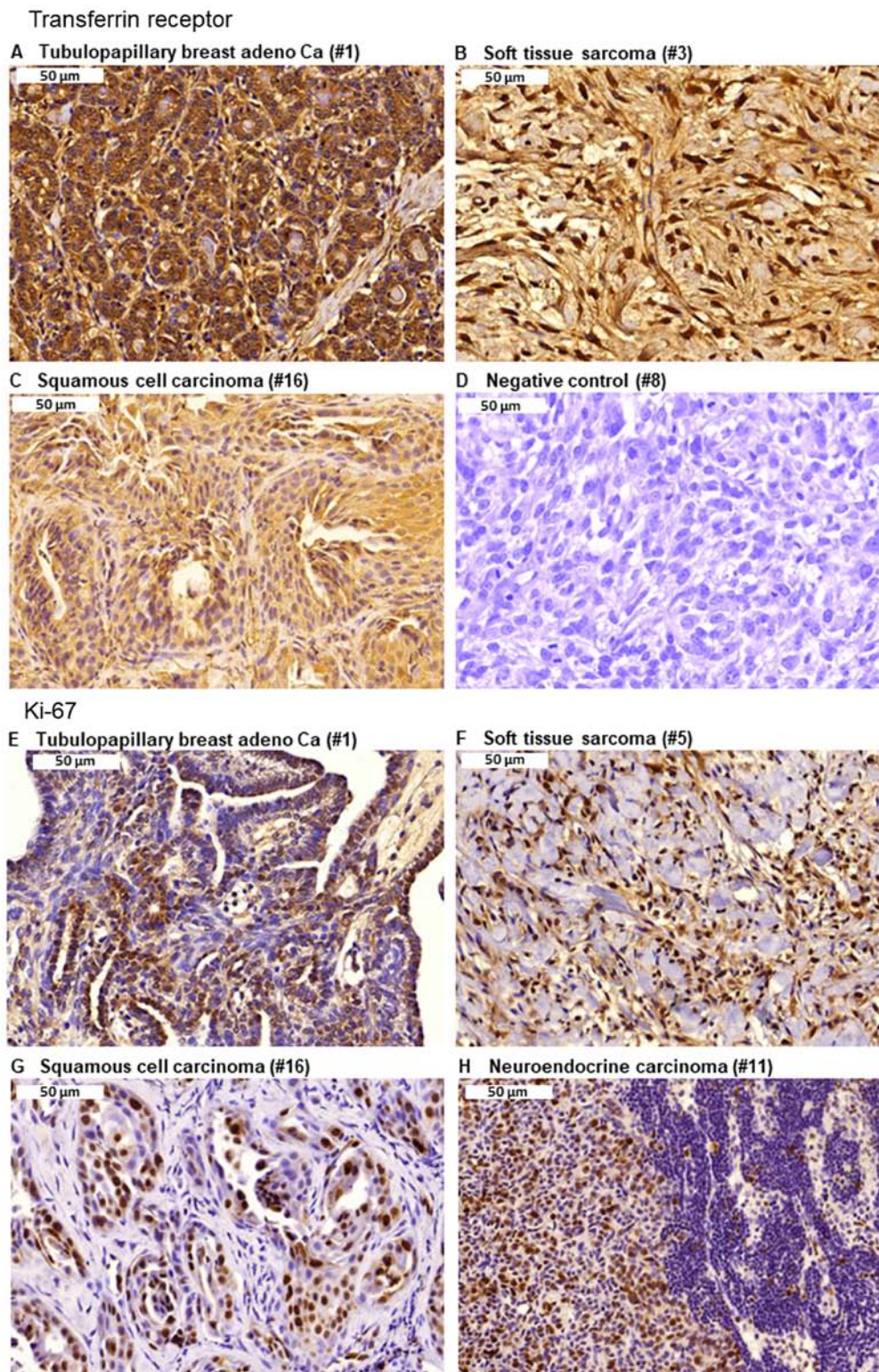
Figure 4. Detection of TfR and Ki-67 expression by immunohistochemistry. (A) TfR in tubulopapillary breast adenocarcinoma. (B) TfR in soft tissue sarcoma. (C) TfR in squamous cell carcinoma. (D) TfR in the negative control. (E) Ki-67 in tubulopapillary breast adenocarcinoma. (F) Ki-67 in soft tissue sarcoma. (G) Ki-67 in squamous cell carcinoma. (H) Ki-67 in neuroendocrine carcinoma. Magnification, x40. TfR, transferrin receptor.

Representative images of immunostaining for TfR expression are presented in Fig. 4A-D. Strong TfR expression was identified in biopsies of tubulopapillary breast adenocarcinoma, soft tissue sarcoma and squamous cell carcinoma. By contrast, the negative control did not demonstrate any reactivity, indicating the specificity of the immunostaining procedure. Furthermore, examples of Ki-67 expression in tubulopapillary breast adenocarcinoma, soft tissue sarcoma, squamous cell carcinoma and neuroendocrine carcinoma are presented in Fig. 4E-H. Furthermore, assessment of the presence of TILs in hematoxylin and eosin stained tumor tissues revealed an absence of TILs in all tumor slides.
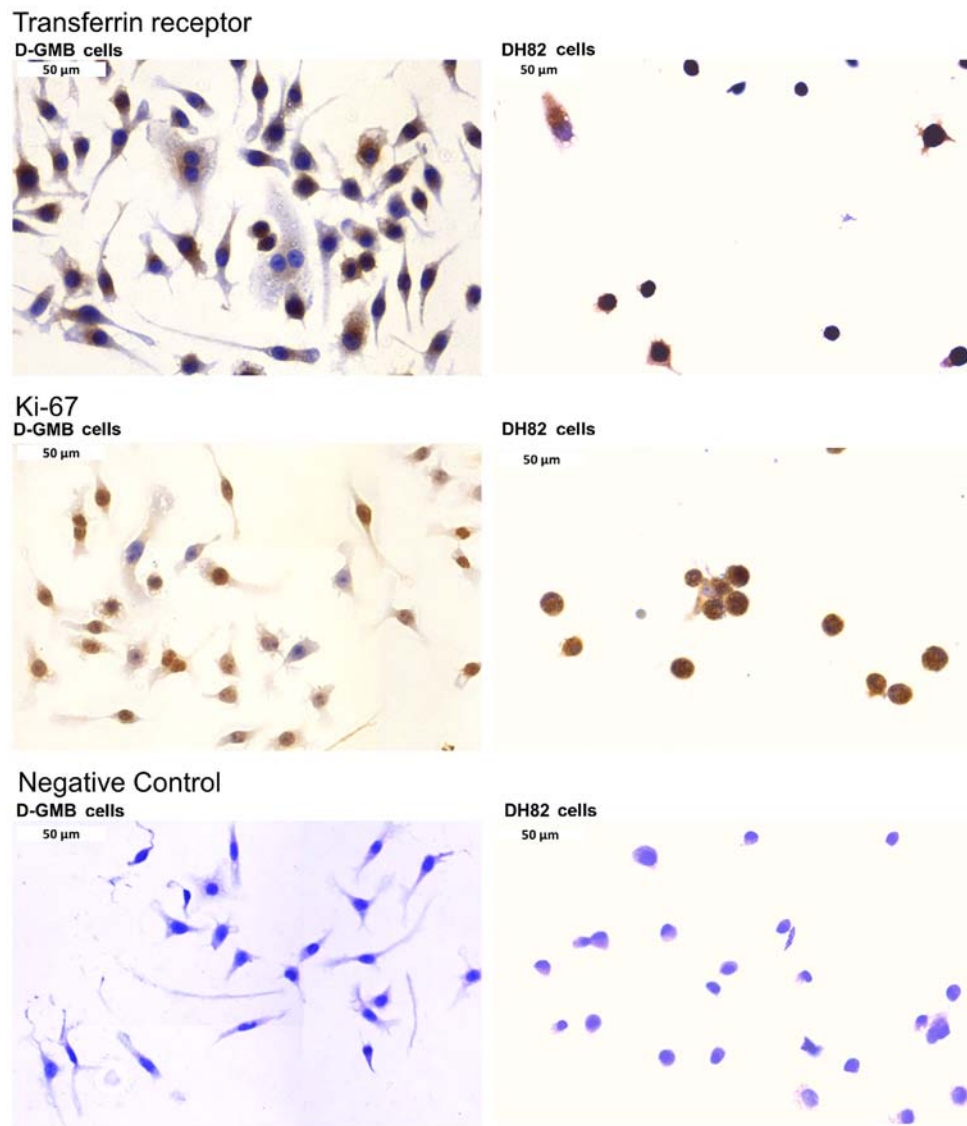
Figure 5. Detection of TfR and Ki-67 expression in dog DH82 histiocytic sarcoma and dog DGMB glioblastoma cell lines. Magnification, x40. TfR, transferrin receptor.

For comparison, the Ki-67 and TfR expression was analyzed in two dog cell lines. DH82 histiocytic sarcoma and DGMB glioblastoma cells were immunostained for both markers. Indeed, Ki-67 and TfR were overexpressed in both cell lines as presented in Fig. 5. Furthermore, the present study investigated the cytotoxicity of artemisinin, artesunate and dihydroartemisinin towards the dog cell lines. As expected, artemisinin and its derivatives were also active in these tumor cell lines. The IC$_{50}$ values for artemisinin, artesunate and dihydroartemisinin towards DH82 cells and DGBM cells are presented in Fig. 6.

Discussion

It has been a matter of discussion among veterinarian physicians and alternative practitioners, whether or not supplementation of iron is beneficial for the activity of artemisinin. The role of heme has been discussed for the antimalarial activity of artemisinin and its derivatives (57,58). In the context of cancer, we previously reported that Ferrosanol® and holotransferrin increase artesunate-induced cytotoxicity and apoptosis in

leukemia and astrocytoma cells up to 10-fold. These effects were reversed by anti-TfR monoclonal antibody RVS10, which competes with transferrin for binding to TfR. While the TfR expression ranged between 48 and 95% in tumor cell lines, normal peripheral mononuclear blood leukocytes revealed ≤1.3% TfR positivity, indicating that artesunate may exert tumor specific effects at least to some extent, because of the preferential TfR expression in tumor cells (59).

In a subsequent study, we investigated a total of 36 cell lines of different tumor types for their response to treatment with artesunate alone or in combination with Ferrosanol® (60). This revealed that artesunate plus Ferrosanol® enhances cytotoxicity compared with artesunate alone in the majority of cell lines; however, 11 cell lines did not demonstrate increased apoptosis and nine lines exhibited a decrease in apoptosis following the combined drug treatment compared with artesunate-treatment alone. It is understood that iron acts as co-factor for proliferation-related enzymes (61). Therefore, Ferrosanol® may induce rather than suppress the proliferation of these nine cell lines. Based on these *in vitro* data, it
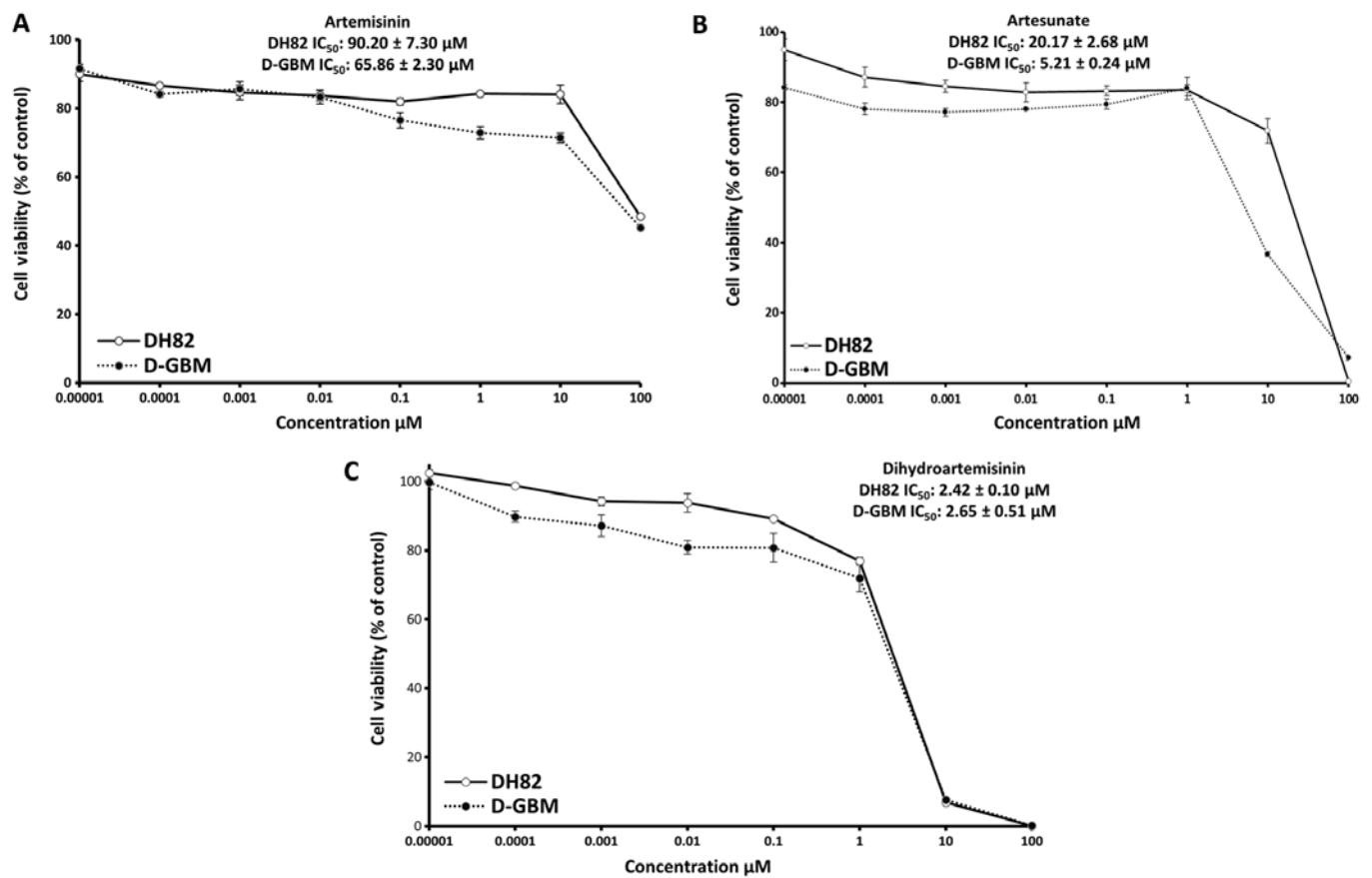
Figure 6. Cytotoxicity towards DH82 and DGMB cells as determined by a resazurin assay. (A) Artemisinin, (B) artesunate and (C) dihydroartemisinin.

cannot be reliably recommended that iron should be added as a supplement to artemisinin-type treatment in veterinary or human cancer therapy. The results of the present study revealed that the blood iron content was not associated with survival times after *A. annua* therapy, while the TfR content inside the tumor was. For practical reasons, it can be assumed that the normal iron content of the body should be sufficient for the activity of artesunate. Only in patients with iron-deficient conditions may iron supplementation be considered. However, in this case it may be important that iron and artesunate reach the tumor at the same time so that a possible enhancing effect of the drug combination can develop. Otherwise, Ferrosanol® may exhibit worse effects and lead to an enhancement rather than a suppression of proliferative tumor activity.

The effect of iron has also been demonstrated in other previous studies. It has been reported that the addition of transferrin can inhibit cross-resistance of multidrug-resistant H69VP small cell lung cancer cells to artemisinin (62). In addition, retinoblastoma cells with high TfR expression are more susceptible to artesunate compared with normal retina cells. This activity is specific, as RNA interference-mediated TfR-knockdown increased the sensitivity of retinoblastoma cells to artesunate (63). In accordance with these findings, supplementation with holotransferrin increases the cytotoxicity of dihydroartemisinin in T-cell lymphoma cells (64).

It is well documented that TfR is more highly expressed in tumor cells compared with normal cells (61,65-68). Therefore,

experimental therapy strategies have been proposed to address TfR as a treatment target to improve tumor-specific killing and spare toxic side effects to normal tissues at the same time, for example by antibody-mediated targeting of TfR or the generation of TfR-directed immunotoxins (69-71). The generation of transferrin-artemisinin conjugates revealed enhanced cytotoxicity towards tumor cells compared with uncoupled artemisinin (72-74).

Previously, we performed a clinical phase I/II trial with artesunate in 23 dogs with cancer, which revealed 1 case of complete remission and 7 cases of stable disease following artesunate-treatment (39). The results of the present retrospective study with *A. annua* appear to be improved. The present study identified an increase in survival time above the threshold of 18 months in 9 of 25 dogs (36%). Although this trend needs to be confirmed in future studies, it could be speculated that the anticancer effect of the whole plant extract is better than that of isolated artemisinin or the semisynthetic derivative, artesunate. Indeed, the plant extract contains many more cytotoxic compounds in addition to artemisinin (75-78), including arteanuin B, artemisitene, scopoletin and 1,8-cineole. Therefore, *A. annua* preparations alone may be used as a combination therapy. Several cytotoxic compounds may act at the same time against the tumor, which leads to an improved tumor inhibition.

It is well understood that rapidly proliferating tumors respond better to standard chemotherapy compared with

slowly growing tumors (79,80). Therefore, the present study also included Ki-67 as proliferation marker in the immunohistochemical analysis. Ki-67 is closely associated with the cell cycle. Its role as a predictive factor for the success of chemotherapy and as a prognostic factor for the survival of cancer patients has been discussed (81,82). Therefore, the significant association between Ki-67 expression and cytotoxicity to artemisinin in the panel of 54 human cell lines from eight different tumor types indicated that Ki-67 expression may also be a prognostic marker for tumor response to artemisinin.

The present study revealed a significant correlation between TfR and Ki-67 expression in veterinary tumors and human tumor cell lines, indicating that TfR expression is associated with high proliferative rates and that artemisinin is more active in fast growing tumors than in slowly growing ones. This result supports previous data from human tumors and also speaks for the comparability of veterinary and human tumors in this respect. The significant correlation between TfR and Ki-67 expression has been reported in biopsies of diverse tumor types, including leukemia, melanoma, breast carcinoma, brain tumors, head and neck cancer and esophageal cancer (83-88). Although a significant association was identified between TfR and Ki-67 in the entire panel of 54 tumor cell lines, this association was not seen in the majority of the subsets with different tumor types. This may simply be explained by the limited number of cell lines per tumor entity. The previously published data on the significant correlation between TfR and Ki-67 expression were obtained in larger collectives of tumor biopsies (83,89).

In human tumor pathology, hematoxylin and eosin staining is also useful for the detection of tumor-infiltrating lymphocytes (TILs) (90-92). Therefore, in the present study we also screened the hematoxylin and eosin-stained slides of the pet tumors for the presence of TILs, but TILs could not be detected in the tumor tissues. Since the tumors were generally large and already progressed at diagnosis, it was assumed that the immune system of the animals was weakened and TIL-mediated immune defenses against the tumor were largely destroyed.

Furthermore, it is important to mention that the present study did not observe considerable adverse side effects among the 25 *A. annua*-treated dogs and cats. In comparison to tumor treatment in human cancer patients with the semi-synthetic drug artesunate, *A. annua* appeared to be even safer. Among the 23 dogs with cancer treated with artesunate, fever and transient hematological and gastrointestinal toxicity were observed in 16 dogs and 1 dog died from pneumonia (39). In human cancer patients, the compassionate use of *A. annua* in 1 patient with prostate carcinoma was well tolerated in a previous study (41). Treatment with artesunate has been reported to lead to occasional and transient side effects, including hematological toxicity, gastrointestinal toxicity, asthenia and thrombosis, in colon, cervix and breast carcinoma (42-44). Rare cases of hepatotoxicity have been reported with artesunate (93,94). Whether phytotherapeutic approaches with *A. annua* are safer than treatment with artesunate requires further investigation.

Notably, the majority of pet owners reported that the animals appeared to feel better after *A. annua* treatment; some were more active, while others were more relaxed. Of course, these observations are subjective and non-quantifiable, and it is unclear whether these reports may reflect the pet owners psychological condition after successful treatment of their pets. Nevertheless, this observation should not be neglected, as it cannot be excluded that there may be an unexpected and unintentional positive side effect of *A. annua*-treatment. Indeed, there are some previous studies that support these observations. The serotonin serum levels in the brains of artesunate-treated rabbits has been reported to be significantly higher compared with in untreated control animals (95). Zhu *et al* (96) reported significant remissions in nociceptive, anxiety and depressive behaviors by dihydroartemisinin, artesunate or artemether. Amos *et al* (97) suggested sedative properties of artemisinin mediated by artemisinin's effect on postsynaptic dopamine D2 receptors in the brain. This novel aspect of artemisinin's possible activity deserves further detailed investigation in the future.

The present investigation also raises the more general question about the comparability of data raised in veterinary tumors to the clinical situation in human tumors and thereby the suitability of veterinary tumors as models of human cancer biology and treatment. The present study determined that the role of TfR and Ki-67 was comparable between veterinary tumors and human tumor cell lines. TfR and Ki-67 have also been associated with response to artemisinin and artesunate in tumors of human patients (41-43). Although these data in human tumors are preliminary, they indicate that veterinary tumors may represent a suitable model for clinical human tumors in the context of artemisinin therapy.

Independent of this situation, there are more arguments that support the suitability of veterinary tumors for the investigation of human cancer biology. Veterinary tumors are spontaneously developing, which may qualify them as better models compared with other tumors in mice and rats. Frequently tumors are maintained by transplanting syngeneic tumors to rodents or human xenograft tumors to immunosuppressed nude mice. Another possibility is to induce tumor development in mice or rats by chemical carcinogens. Although these tumor models are indispensable and of high value in preclinical oncology, they are to some degree artificial. Here, tumors in dogs and cats may be advantageous, because their spontaneous development is closer to the situation of human tumors. Veterinary tumors have not received as much attention as the aforementioned rodent tumor models as of yet. Further investigations are required to investigate the full potential of veterinary tumors as attractive *in vivo* models to develop strategies for human tumor treatment.

In conclusion, the current retrospective study involving 20 dogs and cats treated with standard therapy plus *A. annua* and 11 dogs treated with standard therapy alone clearly demonstrated that additional food supplementation of *A. annua* to veterinary cancer patients resulted in an improved survival prognosis. The activity of *A. annua* may be dependent on the iron content in the tumor, but not in the blood, since TfR expression in the tumors was significantly correlated with survival time and artemisinin cytotoxicity in a control panel of human tumor cell lines. The same was true for Ki-67 expression. Tumors with high Ki-67 expression, indicating high proliferative activity, were more susceptible to artemisinin in the human cell line panel. The data presented in the present study should provide guidance for the activity of *A. annua*

against veterinary tumors. Prospective trials are required to deliver convincing evidence for this hypothesis.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to restrictions of the availability of these data but are available from the corresponding author on reasonable request.

Authors' contributions

TE and EB designed the study. EB treated the animals, provided the material and collected the clinical data. MEMS performed the immunostaining. MEFH performed NMR and HPLC-MS. TE performed the statistical analysis, supervised the work and provided the facilities for the study. TE and MEMS wrote the manuscript. All authors read the manuscript and approved the final version.

Ethics approval and consent to participate

Written permission for this retrospective study was obtained from the *Regierungspräsidium* (Government Presidium) Freiburg, Germany (Az. 35-9185.81/1, dated from February 4th, 2019). Written informed consent for experimental work was obtained from all pet owners.

Patient consent for publication

No applicable.

Competing interests

EB commercially trades Luparte®. No part of the experimental work at the Department of Pharmacological Biology (Johannes Gutenberg University, Mainz, Germany) was funded by EB. All other authors declare that they have no competing interests.

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